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(54) Hybrid molecules having translocation region and cell-binding region.

(57) A hybrid molecule including a first part, a second part and a third part connected by covalent bonds,
(a) the first part including a portion of the binding domain of a cell-binding ligand, which portion is able to cause the hybrid molecule of the invention to bind to an animal cell ;
(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and
(c) the third part including a chemical entity to be introduced into the cell, provided that (i) the hybrid molecule is produced by expression of a recombinant DNA molecule encoding the hybrid molecule, and (ii) the second and third parts are not segments of the same naturally-occurring polypeptide toxin ;
a recombinant DNA molecule encoding the hybrid molecule ; and
a method of producing the hybrid molecule.

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HYBRID MOLECULES HAVING TRANSLOCATION REGION AND CELL-BINDING REGION

Background of the Invention

This invention relates to hybrid molecules having a cell-binding part and a translocation part.

The literature contains many examples of fused genes which code for hybrid proteins. For example, Villa-Komaroff et al., Proc. Natl. Acad. Sci. U.S.A. 75 : 3727-3731, 1978, describes a fused gene made up of a eukaryotic structural gene fused to a non-cytoplasmic bacterial gene. The fused gene codes for a hybrid protein which is transported out of the cytoplasm.

Hybrid proteins also have been made by other methods (e.g., the coupling of two different protein molecules) which do not involve recombinant DNA techniques. For example, it has been proposed to form, by coupling, therapeutic hybrid proteins consisting of portions of toxin molecules coupled to a ligand capable of binding specifically to a selected class of cells. One attempt to make such a hybrid protein, reported in Chang et al., J. Biol. Chem. 252 :1515-1522, 1977, resulted in a hybrid consisting of the diphtheria toxin A chain coupled to human placental lactogen hormone by cross-linking through a disulfide bond. Although the hybrid protein bound to cells containing lactogen receptors, it did not inhibit protein synthesis in those cells.

A hybrid protein consisting of the ricin toxin A chain coupled to the β chain of human chorionic gonadotropin hormone by similarly cross-linking through a disulfide bond has also been reported ; although said to have specifity, its binding capacity has not been reported. Furthermore, extremely high concentrations were required to significantly inhibit protein synthesis in rat Leydig tumor cells, making it difficult to distinguish between "non-specific" entry caused by endocytosis and "specific" entry caused by transport of the toxic portion of the hybrid across the cytoplasmic membrane of the target cells (Oeltman et al., J. Biol. Chem. 254 :1028-1032, 1979). The same shortcoming was found in a hybrid protein consisting of diphtheria A coupled to insulin using cystamine as the cross-linking agent (Miskimins et al., Biochem. Biophys. Res. Commun. 91 :143-151, 1979). A hybrid consisting of ricin A coupled to epidermal growth factor (EGF) by means of a heterobifunctional cross-linker has also been made ; the binding characteristics provided by the EGF are not limited to specific cells, but rather encompass a wide variety of cell types (Cawley et al., Cell 22 :563-570, 1980).

As illustrated in Fig. 1, the natural diphtheria toxin molecule consists of several functional "domains" which can be characterized, starting at the amino terminal end of the molecule, as hydrophobic leader signal sequence s (amino acids Val_{-25} - Ala_{-1}) ; enzymatically-active Fragment A (amino acids Gly_1 - Arg_{193}) ; the protease-sensitive disulfide loop I_1 (amino acids Cys_{186} - Cys_{201}), containing a cleavage domain ; and Fragment B (amino acids Ser_{194}-Ser_{535}), which includes a translocation domain and a generalized binding domain flanking a second disulfide loop (I_2, amino acids Cys_{461} - Cys_{471}).

The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps : (i) the binding domain of diphtheria toxin binds to specific receptors on the surface of a sensitive cell ; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle ; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage in I_1 between fragments A and B ; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin spontaneously inserts into the endosomal membrane ; (v) once embedded in the membrane, the translocation domain of the toxin facilitates the delivery of Fragment A into the cytosol ; (vi) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide - dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the intoxicated cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to shut down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by Pseudomonas exotoxin A, and possibly by certain other naturally-occurring toxins, is very similar.

Summary of the Invention

In general, the invention features, in one aspect, a hybrid molecule including a first part, a second part, and a third part connected by covalent bonds,

(a) the first part including a portion of the binding domain of a cell-binding ligand, which portion is able to cause the hybrid molecule of the invention to bind to an animal cell ;

(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and

(c) the third part including a chemical entity (here, a polypeptide) to be introduced into the cell, provided that (i) the hybrid molecule is produced by expression of a recombinant DNA molecule encoding the hybrid molecule, and (ii) the second and third parts are not segments of the same naturally-occurring polypeptide

toxin, (although the first and second parts may be derived from the same toxin : e.g., diphtheria toxin or Pseudomonas exotoxin A). "Translocation" here means the facilitation of movement of a chemical entity from the exterior surface of a cellular membrane (or what constituted the exterior surface prior to formation of an endocytic vesicle), through the membrane, and into the cytosol at the interior of the cell. A "translocation domain" is a segment of a protein which, when the protein is bound to the exterior surface of a cellular membrane, is capable of translocating some portion of that protein through the membrane.

The invention also features a hybrid molecule including a first part, a second part, and a third part connected by covalent bonds,

(a) the first part including a portion of the binding domain of a cell-binding ligand (which may be a polypeptide ligand or may be another type of ligand such as a steroid hormone), which portion is effective to cause the hybrid molecule to bind to a cell of an animal ;

(b) the second part including a portion of a translocation domain of a protein, which portion is capable of translocating the third part across the cytoplasmic membrane of the cell ; and

(c) the third part including a chemical entity (which may or may not be a polypeptide) to be introduced into the cell, provided that (i) the first and second parts are not segments of the same naturally-occurring polypeptide toxin, and (ii) likewise, the second and third parts are not segments of the same naturally-occurring polypeptide toxin.

In preferred embodiments, the second part of either type of hybrid molecule described above comprises at least a portion of the translocation domain of a naturally-occurring toxin (e.g. diphtheria toxin or Pseudomonas exotoxin A), and the ligand comprises a hormone (e.g., a polypeptide hormone such as insulin, Interleukin II (also termed "IL2"), Interleukin IV, Interleukin VI or EGF) ; an antigen-binding, single-chain analog of a monoclonal antibody ; or a polypeptide toxin capable of binding to the desired class of cells. Where all three parts are polypeptides, the hybrid molecule is preferably a recombinant protein (that is, a protein produced by recombinant DNA techniques). More preferably, the third part is an antigen-binding, single-chain analog of a monoclonal antibody (where such antigen is, for example, a viral protein such as the human immunodeficiency virus (HIV) protease), or alternatively, the enzymatically active portion of an enzyme (e.g., hexosaminidase A; α-1,4-glucosidase ; phenylalanine hydroxylase ; a protease ; a nuclease ; or a toxin such as cholera toxin, LT toxin, C3 toxin, Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin or Pseudomonas exotoxin A), and most preferably it supplies an enzymatic activity in which the cell is deficient : as, for example, in the case of a genetic deficiency. Where the enzyme is cholera toxin, the resulting hybrid molecule may be used to raise the cyclic AMP level within a target animal cell : preferably, the target cell so treated is a T-cell and the hybrid molecule includes at least a portion of the binding domain of IL2. The hybrid molecule of the invention includes the cholera toxin A/diphtheria toxin B'/IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 6 ; the Shiga-like toxin A/diphtheria toxin B'/ IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 9 ; the ricin A/diphtheria toxin B'/IL2 hybrid polypeptide encoded by the plasmid illustrated in Fig. 12 ; the phenylalanine hydroxylase/diphtheria toxin fragment B hybrid polypeptide encoded by the plasmid illustrated in Fig. 14 ; an HIV protease-binding protein (HIVP-BP)/diphtheria toxin B'/IL2 hybrid polypeptide prepared as hereinafter described ; and a Shiga-like toxin A/IL2 hybrid in which both the enzymatic activity and the translocation function are provided by the Shiga-like toxin A portion of the hybrid, and which contains a proteolytically-sensitive disulfide loop. Also included are biologically active mutational analogs of any of the above hybrid polypeptides. As used herein, a "biologically active mutational analog" is a polypeptide which exhibits the same type of cell-binding specificity and the same type of biological activity (e.g., a particular enzymatic or antigen-binding activity) as the listed hybrid polypeptide of which it is an analog, but which differs from such listed hybrid polypeptide by one or more deletions and/or one or more substitutions of segments of one or more amino acid residues. Preferably, the amino acid sequence of the biologically active mutational analog shows at least a 70% (more preferably 80% and most preferably 90%) homology (i.e., identity of amino acid sequence) with the hybrid polypeptide of which it is an analog, and the analog exhibits at least 50% (more preferably, at least 75%) of a biological activity exhibited by the hybrid polypeptide of which it is an analog.

Also within the invention is a recombinant DNA molecule encoding any of the above hybrid polypeptide molecules (including biologically active mutational analogs), a vector including such a recombinant DNA molecule, a cell containing such a vector or recombinant DNA molecule (and which preferably is capable of expressing the recombinant DNA molecule to produce the hybrid polypeptide encoded by it), and a method of preparing the hybrid polypeptide molecule of the invention by permitting a cell containing a recombinant DNA molecule encoding the polypeptide (the "transformed cell") to express the recombinant DNA molecule.

In other preferred embodiments, the third part comprises a detectable label, more preferably a fluorescent moiety, a radioactive moiety, or an electron-dense moiety.

The invention also features a method of labeling a class of cells, which method involves contacting the cells with a hybrid molecule having a third part comprising a detectable label.

Also included in the invention are (1) a method of treating an animal having a deficiency in a certain enzyme, by administering to the animal an effective amount of a hybrid molecule comprising that enzyme ; and (2) a method of treating a human patient infected with HIV, by administering to the patient an effective amount of a hybrid molecule having as its third part an HIV protease-binding, single-chain analog of a monoclonal antibody against HIV protease.

Based upon the observation that certain types of polypeptide toxins have three separate functional regions, one region which binds the molecule to particular receptors on the surface of a target cell, a second one which facilitates entry of the enzymatically-active region into the cytosol of the cell, and a third region which exhibits the enzymatic activity that characterizes the toxic effect of the molecule, the invention includes tripartite hybrid molecules in which any of these regions may be replaced with functionally comparable regions from other sources. That is, the first functional region may be replaced with a particular binding moiety which binds the hybrid molecule to a selected class of cells, such as IL2 (which binds to high-affinity IL2 receptor-bearing T-cells), or α melanocyte stimulating hormone (αMSH, which binds to melanocytes), or a moiety which binds to a broad spectrum of cell types, as is characteristic of the binding domains of cholera toxin and diphtheria toxin ; the second part may be taken from any type of polypeptide in which a translocation domain is identifiable, but will most likely be from a toxin molecule that translocates in a manner similar to diphtheria toxin and Pseudomonas exotoxin A. The third part may be any type of moiety that one wants to insert into the cell and that will fit through the channel in the membrane formed by the translocation domain : for example, a cell-killing enzyme such as Shiga toxin ; a metabolic enzyme such as phenylalanine hydroxylase (the enzyme in which phenylketonurics are deficient) ; an antigen-binding, single-chain analog of a monoclonal antibody against an antigen that appears within the target cell ; or a fluorescent label. A proteolytically-sensitive loop (such as I_1 of diphtheria toxin) between the enzymatically-active region of the hybrid and the remainder of the hybrid appears to play an important role in the hybrid's function.

Although the medical community is rapidly expanding its understanding of the molecular bases of many diseases, one problem has particularly frustrated efforts to translate this understanding into rational protocols for treating the diseases : the problem of how to direct the appropriate therapy into the affected cells so that it can function properly to alleviate or cure the disease. By providing such a method, the present invention will have virtually unlimited applications : from treating genetic deficiency diseases by delivering to affected cells an enzyme supplying the missing function, to supplementing cellular levels of a particular enzyme or a scarce precursor or cofactor, to directing toxins or other poisons to destroy particular cells (such as adipocytes, cancer cells, or virus-infected cells), to counteracting viral infections such as HIV (which causes Acquired Immunodeficiency Syndrome ("AIDS")) by introducing into appropriate cells antibodies to viral proteins. The invention also provides a means for getting other, non-therapeutic substances, such as detectable labels, into targeted cells. The use of a translocation mechanism ensures that the hybrid will be effective in relatively low doses, since a high proportion of the substance of interest will be taken into the targeted cells.

To the extent that the three parts of the hybrids of the invention are polypeptides, they may be manufactured as a single hybrid recombinant protein, permitting reproducibility, consistency, and the precise control of composition which is desirable for any pharmaceutical product.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will be briefly described.

Drawings

Fig. 1 is a diagrammatic representation of the diphtheria toxin molecule.

Fig. 2 is a restriction map showing the location and orientation of the diphtheria tox gene on the 3.9 kb BamHI restriction fragment of corynephage β^{tox}.

Fig. 3 is a representation of the diphtheria toxin gene and flanking regions, with the protein encoded shown above ; the B′ region is the region between the labeled Sau3A1-2 and SphI sites.

Fig. 4 is a diagrammatic representation of the strategy used to construct a plasmid encoding fragment B′ of diphtheria toxin.

Fig. 5 is a representation of the nucleotide sequence of the Vibrio cholerae toxin gene, with amino acids shown below corresponding codons.

Fig. 6 is a diagrammatic representation of the cloning strategy followed to construct a plasmid encoding cholera toxin A_1-diphtheria toxin B′- IL2 hybrid.

Fig. 7 is a diagrammatic representation of the cloning strategy followed in order to construct the plasmid pPA123.

Fig. 8 is a representation of the nucleotide sequence of the E.coli bacteriophage H19B Shiga-like toxin gene, with amino acids shown below corresponding codons.

Fig. 9 is a diagrammatic representation of a cloning strategy proposed for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid.

Fig. 10 is a diagrammatic representation of an alternative cloning strategy for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid.

Fig. 11 is a representation of the nucleotide sequence of the Ricinus communis ricin gene, with amino acids shown above corresponding codons ; this figure is adapted from Fig. 2 of Halling et al. (Nucl. Acids Res. 13 :8019-8033, 1985).

Fig. 12 is a diagrammatic representation of the cloning strategy proposed for constructing a plasmid encoding a ricin A-diphtheria toxin B'-IL2 hybrid.

Fig. 13 is a representation of the nucleotide sequence of human phenylalanine hydroxylase cDNA, with amino acids shown below corresponding codons.

Fig. 14 is a diagrammatic representation of a cloning strategy proposed for constructing a plasmid encoding a phenylalanine hydroxylase-diphtheria toxin B hybrid.

Structure

One embodiment of the hybrid molecule of the invention is a three-part hybrid protein which includes (1) a cell-binding domain ; (2) a translocation domain, such as that of diphtheria toxin ; and (3) a polypeptide to be introduced into the cell, which polypeptide is not derived from the same naturally-occurring protein as is the translocation domain sequence. The cell-binding domain may be generalized (i.e., it is capable of binding the hybrid molecule to a wide variety of cell types, like the cell-binding domain of naturally-occurring diphtheria toxin), or specific for one or a few types of cells. The third part of the hybrid is covalently linked to the translocation domain, such that the translocation domain is capable of translocating the third part into or across the membrane of the cell to which the cell-binding portion of the hybrid is bound. This third part may be, for example, an enzymatically active polypeptide, an antigen-binding portion of a monoclonal antibody, or a detectable label such as a fluorescent dye. It may not, however, be a fragment of the same naturally-occurring molecule from which the translocation domain originates.

Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin and Pseudomonas exotoxin A, and may include other toxins and non-toxin molecules, as well. The translocation domains of diphtheria toxin and Pseudomonas exotoxin A are well characterized (see, e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82 :1692-1696, 1985 ; Colombatti et al., J. Biol. Chem. 261 :3030-3035, 1986 ; and Deleers et al., FEBS 160 :82-86, 1983), and the existence and location of such a domain in other molecules may be determined by methods such as those employed by Hwang et al., Cell 48 :129-136, 1987 ; and Gray et al., Proc. Natl. Acad. Sci. USA 81 :2645-2649, 1984.

The segment of diphtheria toxin labelled "Fragment B" in Fig. 3 includes both the translocation domain and the generalized cell-binding domain of the naturally-occurring molecule. Truncation of Fragment B to the segment marked B' effectively eliminates the cell-binding function of diphtheria toxin while retaining the translocation function of the molecule. A portion of Fragment B encoded by a sequence ending at or downstream from the Sph1 restriction site may be used for the translocation domain ; however, if the hybrid includes a cell-binding region other than that of diphtheria toxin, the portion of Fragment B downstream from the Sph1 site may be included only if it does not include sequences encoding a sufficient part of the diphtheria toxin receptor-binding domain to yield a functional diphtheria toxin receptor-binding domain.

The part of the hybrid protein contributed by the polypeptide ligand can consist of the entire ligand, or a portion of the ligand which includes the entire binding domain of the ligand, or an effective portion of the binding domain. When the ligand being used is large, it is desirable that as little of the non-binding portion as possible of the ligand be included, so that the binding domain of the molecule is positioned close to the translocation domain. It is also desirable to include all or most of the binding domain of the ligand molecule.

The polypeptide portions of the hybrids of the invention are conveniently made using recombinant DNA techniques involving forming the desired fused gene encoding the hybrid protein, and then expressing the fused gene. Chemical cross-linking is utilized only where one or more of the parts of the hybrid molecule are not polypeptides.

Standard procedures for DNA cloning, cell transformation and plasmid isolation (as described, for example, by Maniatis et al., Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), and for oligodeoxynucleotide synthesis, could be employed to carry out the following construc-

tions.

Example 1. Fusion of a gene fragment encoding diphtheria toxin B' with sequence encoding cell-binding portions of various ligands ; labeling and use of the resultant hybrid polypeptides

Referring to Figs. 2 and 3, the location and orientation of the diphtheria tox operon on the 3.9 kb BamHI restriction fragment of corynephage β^{tox+} allows the tox operon to be cleaved at a desired location, and the desired portion of the operon to be fused with the desired portion of the gene for a selected polypeptide ligand.

Gene fusions of the invention may be made as follows. First, the NsiI-SphI fragment encoding most of Fragment B' (Fig. 3) is isolated from the tox gene of plasmid pABM508 (Murphy et al., Proc. Natl. Acad. Sci. USA 83 :8258-8262, 1986). The following linker is ligated into the 5' (NsiI) end :

```
5'      C ATG TCA GTA GGT AGC TCA TTG TCA TGC A  3'
3'          AGT CAT CCA TCG AGT AAC AGT          5'
encoding fmet-Ser-Val-Gly-Ser-Ser-Leu-Ser-Cys
         1/2                                1/2
         NcoI                               NsiI
```

and the following linker is ligated into the 3' (SphI) end :

```
5'          CAT GAA        3'
3'  G TAC GTA CTT TCG A    5'
      1/2           1/2
      SphI          HindIII
```

The resulting fragment is then cloned into NcoI + HindIII-digested pKK233-2 (Pharmacia, Piscataway, NJ). This modification allows the expression of Fragment B' to be driven off the trc promoter (P_{trc}) in E.coli. The SphI site permits in-frame fusion with a gene sequence encoding the binding domain of a peptide ligand.

Generally, the manipulative operations are carried out using cloning vectors ; e.g., phages or plasmids. The genetic material coding for the binding domain of the polypeptide ligand can be either DNA cloned from a natural source, or a synthetic oligonucleotide sequence. Generally the fused gene will reside on a cloning vector, e.g., a plasmid or a phage, which is used to transform cultured bacteria, yeast or tissue culture host cells. The hybrid protein is then harvested from the cells using conventional techniques. Purification of the hybrid proteins of the invention, regardless of the polypeptide ligand used, can be carried out via affinity chromotography, using a monoclonal antibody against diphtheria toxin Fragment B.

The purified hybrid protein of the invention may be used as a transport system to carry a detectable label into specific cells. The label which is attached to the hybrid protein molecule can be any conventional atom or molecule used for diagnostic labeling ; examples are radioactive labels such as ^{125}I-compounds, technitium isotopes, NMR reporter groups, and fluorescent dyes. The most preferred labels are hydrophobic labels such as fluorescent dyes (most conventional fluorescent dyes happen to be hydrophobic) which are incorporated into the cytoplasmic membrane of target cells, as will be explained in more detail below. Labels can be attached to the hybrid protein according to conventional labeling techniques. Labels are used in an amount (e.g., one or two label molecules per protein molecule) which does not interfere with the binding or cell penetration functions of the hybrid protein molecule.

The labeled hybrid proteins of the invention can be used diagnostically to label a desired class of target cells, a class determined by the specific polypeptide ligand which imparts the binding domain of the hybrid molecule. The specific binding domain of the polypeptide ligand portion selectively binds to those cells ; the labeled molecule is then taken up by the cells via receptor-mediated endocytosis, and the label subsequently is delivered to the cell membrane and/or the cytoplasm of the target cells.

The process by which a labeled hybrid protein of the invention can be incorporated into cells can be summarized as follows. The labeled hybrid protein is taken up by target cells via receptor-mediated endocytosis into an endocytic vesicle ; thereafter a pH differential across the membrane of the endocytic vesicle is established as a result of the cell's ATP-dependent proton pump. The pH differential across the membrane causes the hybrid protein, including its lipid-associating portion and its label, to be inserted into the plane of the membrane of the endocytic vesicle. The hydrophobic nature of the hybrid protein causes it to remain in the membrane, protected from the rapid enzymatic degradation which would occur were the labeled protein to reside in the cytoplasm or in the lumen of the endocytic vesicle.

After insertion into the plane of the membrane of the endocytic vesicle, the labeled hybrid protein can "traf-

fic", as follows. The endocytic vesicle buds off from the cytoplasmic membrane and enters the cytoplasm of the cell, where it can merge with a lysosome into which the labeled hybrid protein is then incorporated. Alternatively, the endocytic vesicle can recycle to the cytoplasmic membrane of the cell. In either case, the label remains trapped in the target cell.

As is mentioned above, a major diagnostic use of the labeled hybrid proteins will be the in vivo and in vitro detection of metastatic loci, using conventional cell staining and labeling techniques. Such detection could be of particular value in surgery, by providing the surgeon with information needed to know how much tissue to excise when removing, e.g., metastatic melanoma cells.

Example 2. Construction of a cholera toxin A_1-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein

Plasmid pCVD2 containing the coding sequence for the enzymatically-active A_1 fragment of cholera toxin (see Fig. 5) was prepared from a Vibrio cholera DNA library as described by Mekalanos et al. (Nature 306 :551-557, 1983). Fig. 6 outlines the strategy employed in engineering a cholera toxin A_1-diphtheria toxin B'-IL2 gene. Briefly, pCVD2 was cleaved with the restriction enzyme XbaI at the unique XbaI site. The following synthetic linker, which has 1/2 of an XbaI site at each end, was ligated to the linearized plasmid in order to introduce a NcoI site upstream from the XbaI site :

```
          5' C TAG ACC ATG GGA AAT GAT GAT AAG TTA-
          3'     TGG TAC CCT TTA CTA CTA TTC AAT-
peptide:           fmet-Gly-Asn-Asp-Asp-Lys-Leu-
                1/2
               XbaI
```

```
(cont.)    - TAT CGG GCA GAT T      - 3'
           - ATA GCC CGT CTA AGA TC - 5'
           - Tyr-Arg-Ala-Asp-Ser-Arg
                                1/2
                               XbaI
```

The appropriate construct was selected by restriction site mapping and sequence determinations, and then was digested with Nco1 and ClaI to produce a Nco1-ClaI fragment. This in turn was digested with ScrFI. The 3' end of the resulting Noc1-ScrFI fragment was ligated to the following synthetic linker :

```
          5' -  G GGT TCA GGG CC - 3'
          3' -    CCA AGT C      - 5'
peptide:  Pro-Gly-Ser-Gly-Pro
          1/2               1/2
          ScrFI             ApaI
```

The polypeptide encoded by the resulting NcoI-ApaI fragment lacks the natural cholera toxin signal sequence, having instead fmet-Gly followed by the mature A_1 region of cholera toxin, followed by Gly-Ser-Gly-Pro. This construct can be cloned into a plasmid that encodes diphtheria toxin fragment B' fused to the human interleukin-2 gene (plasmid pPA123, Fig. 7). Plasmid pPA123 was constructed from plasmid pDW124 (Diane Williams, Ph.D. dissertation, Boston University School of Medicine, Department of Microbiology, Boston, MA, 02118, 1989) as outlined in Fig. 7. Plasmid pDW124 encodes a diphtheria toxin fragment A-fragment B'-IL2 fusion protein that is expressed off the trc promoter in E.coli. The sequences encoding fragment A were deleted by digestion with the restriction endonucleases NcoI and NsiI. The following oligonucleotides were used to rebuild the fragment A/B disulfide loop (I_1) sequence, introduce an ApaI site on the 5' end of the loop, and recreate the NcoI site encoding the translation-initiating ATG codon :

```
5' -      C ATG GGG TCA GAT GGG CCC TGT GCA GGA AAT CGT GTC-
3' -            CC AGT CTA CCC GGG ACA CGT CCT TTA GCA CAG-
peptide:  fmet-Gly-Ser-Val-Gly-Pro-Cys-Ala-Gly-Asn-Arg-Val-
          1/2                   ApaI
          NcoI
```

```
(cont.)     -AGG CGA TCA GTA GGT AGC TCA TTG TCA TGC A - 3'
            -TCC GCT AGT CAT CCA TCG AGT AAC AGT       - 5'
            -Arg-Arg-Ser-Val-Gly-Ser-Ser-Leu-Ser-Cys
                Sau3AI                          1/2
                                                NsiI
```

Plasmid pPA123 resulted from ligating the above oligonucleotide fragment onto the NcoI-NsiI-digested pDW124 vector fragment. Plasmid pPA123 can now be used to fuse sequences encoding cholera toxin fragment A_1 to diphtheria toxin B'-IL2 as shown in Fig. 6. Plasmid pPA123 is digested with restriction enzymes NcoI and ApaI, and the resulting vector fragment is ligated to the modified cholera toxin fragment A_1 described above, to yield a plasmid encoding a cholera toxin A_1-diphtheria toxin B'-IL2 hybrid ("CTA/DTB'/IL2 hybrid"), which is expressed from the tcr promoter on the plasmid.

Following expression of the recombinant gene in E.coli, the CTA/DTB'/IL2 hybrid protein can be isolated and used in appropriate treatment regimens : for example, as an adjunct to treatment with diphtheria toxin-IL2 hybrid. Diphtheria toxin-IL2 hybrid effectively targets the cell-killing ability of diphtheria toxin to cells bearing the IL2 receptor, such as certain leukemic T-cells. However, the pharmacological effectiveness of diphtheria toxin-IL2 hybrid is diluted by circulating endogenous IL2, which is naturally synthesized by activated T-cells and which competes with diphtheria toxin-IL2 hybrid for IL2 receptors on T-cells. By first exposing the target cells to CTA/DTB'/IL2 hybrid, the biological activity of cholera toxin can be harnessed to alleviate this problem. The A_1 subunit of natural cholera toxin enzymatically catalyzes the ADP-ribosylation of a GTP-binding regulatory component of the adenylate cyclase complex, resulting in the accumulation of cyclic AMP within the affected cell and thereby disrupting a multitude of cellular functions without killing the cell. Targeting the cholera toxin A_1 activity specifically to cells bearing the IL2 receptor will result in the temporary inhibition of IL2 synthesis within those cells. This permits depletion of the amount of circulating IL2 available to compete with diphtheria toxin-IL2 for IL2 receptors, without interfering with expression of IL2 receptors on the surfaces of the T-cells and without injuring non-targeted cells. Subsequent treatment with diphtheria toxin-IL2 will thus be more effective at killing T-cells than if CTA/DTB'/IL2 hybrid had not been used.

Example 3. Construction of a Shiga-like toxin A-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein

The DNA sequence and corresponding amino acid sequence for the A subunit of Shiga-like toxin ("SLT-A") are shown in Fig. 8. Bacteriophage H19B DNA from a strain of E.coli that produces SLT-A is prepared as described by Calderwood et al. (Proc. Natl. Acad. Sci. USA 84 :4364-4368, 1987) and digested with TaqI and XmnI. A TaqI-XmnI fragment (approx. 650 bp) corresponding to most of the coding sequence for SLT-A (the "sltA gene") is isolated therefrom (see Fig. 9) ; the following oligonucleotide is then ligated onto the 5'(TaqI) end of the fragment :

```
      5' - CATG GGA AAG GAA TTT ACC TTA GAC TTC T   - 3'
      3' -      CCT TTC CTT AAA TGG AAT CTG AAG AGC - 5'
peptide: fmet-Gly-Lys-Glu-Phe-Thr-Leu-Asp-Phe-Ser
         1/2                                 1/2
         NcoI                                TaqI
```

This oligonucleotide sequence provides an fmet-Gly coding sequence followed by a sequence coding for the first eight amino acids of the mature SLT A subunit, to replace the section of the natural gene (coding for the toxin signal peptide and same eight amino acids of the mature SLT A subunit) which was cleaved off during TaqI digestion of the gene. Also provided by the oligonucleotide linker is a 1/2 NcoI site at the 5' end of the construct, to permit expression from the trc promoter of the hybrid plasmid.

The following oligonucleotide sequence, which regenerates the coding region (cleaved off by XmnI diges-

tion) for the carboxyl end of the SLT A subunit up to the initial Cys codon, and introduces an ApaI restriction site, is ligated to the 3' (XmnI) end of the sltA gene fragment :

```
          5' - ATT TCT TTT GGA AGC ATT AAT GCA ATT CTG-
          3' - TAA AGA AAA CCT TCG TAA TTA CGT TAA GAC-
peptide: Ile-Ser-Phe-Gly-Ser-Ile-Asn-Ala-Ile-Leu-
                1/2
                XmnI

(cont.) -GGA AGC GTG GCA TTA ATA CTG AAT GGG CC -3'
        -CCT TCG CAC CGT AAT TAT GAC TTA C      -5'
        -Gly-Ser-Val-Ala-Leu-Ile-Leu-Asn-Gly-Pro
                                    1/2 ApaI
```

The NcoI-ApaI sltA gene sequence can be ligated into a NcoI + ApaI-digested plasmid pPA123 (Fig. 7) to yield a SLTA-diphtheria toxin B'-IL2 ("SLTA/DTB'/IL2 hybrid") gene that can be expressed in E.coli from the trc promoter on the plasmid (see Fig. 9).

An alternative cloning strategy for constructing a plasmid encoding a Shiga-like toxin A-diphtheria toxin B'-IL2 hybrid is illustrated in Fig. 10.

Purified SLTA/DTB'/IL2 hybrid protein would be useful as a treatment for conditions involving overproduction of cells bearing IL2 receptors, such as certain T-cell lymphomas and organ transplant rejection crises. As is the case for diphtheria toxin-IL2, the IL2 portion of the hybrid causes the hybrid to attach specifically to IL2-receptor-bearing cells, so that the enzymatic portion of the hybrid can be inserted into the targeted cell ; the enzymatic portions of both types of hybrid toxins then act on the protein synthesis machinery in the cell to shut down protein synthesis, thus killing the cell. The difference between these two types of hybrid toxins is the nature of their enzymatic activities : the enzymatic portion of diphtheria toxin-IL2 hybrid catalyzes the ADP-ribosylation by nicotinamide adenine dinucleotide of Elongation Factor 2, thereby inactivating this factor which is necessary for protein synthesis, while the enzymatic portion of SLTA/DTB'/IL2 hybrid is a ribonuclease capable of cleaving ribosomal RNA at a critical site, thereby inactivating the ribosome. SLTA/DTB'/IL2 hybrid would therefore be useful as a treatment for the same indications as diphtheria toxin-IL2 hybrid, and could be substituted if, for example, the proliferating T-cells develop a resistance to the latter hybrid toxin.

Example 4. Construction of ricin A-diphtheria toxin B'- IL2 gene, and use of the resultant hybrid protein

A genomic clone bank of castor bean (Ricinus communis) DNA is prepared as described in Halling et al., Nucl. Acids Res. 13 :8019-8033, 1985, and a ~780bp BanI fragment of the ricin gene, corresponding to most of the ricin A domain (the enzymatic domain) and a portion of the ricin A-to-B linker peptide, is isolated therefrom (see Fig. 11). The following synthetic oligonucleotide is ligated onto both ends of the fragment, phosphorylating only the bottom strand of DNA shown :

```
      5' -     C ATG GCT ATA TTC CCC AAA CAA TAC CCA ATT-
      3' -         CGA TAT AAG GGG TTT GTT ATG GGT TAA-
      Peptide:  fmet-Ala-Ile-Phe-Pro-Lys-Gln-Tyr-Pro-Ile-
             1/2
             NcoI

(cont.)  -ATA AAC TTT ACC ACA GCG G      - 3'
         -TAT TTG AAA TGG TGT CGC CCA CG - 5'
         -Ile-Asn-Phe-Thr-Thr-Ala-Gly-Ala
                                  1/2
                                  BanI
```

The resulting ligated fragment (illustrated in Fig. 11) is partially digested with FspI, and the ~780bp band corresponding to a BanI-FspI ricin A gene fragment with a NcoI-BanI linker at the 5' end is isolated (see Fig. 11). The NcoI-BanI linker supplies the mature ricin A N-terminal amino acid codons which were cleaved from the

fragment during BanI digestion, as well as the codons for fmet-Ala to replace the natural ricin A signal peptide.

The following oligonucleotide is ligated onto the 3' (FspI) blunt end of the fragment, phosphorylating only the top strand shown :

```
        5' - GCA CCT CCA CCA TCG TCA CAG TTT GGG CC - 3'
        3' - CGT GGA GGT GGT AGC AGT GTC AAA C      - 5'
  Peptide:  Ala-Pro-Pro-Pro-Ser-Ser-Gln-Phe-Gly-Pro
            1/2                                 1/2
            FspI                                ApaI
```

This linker supplies the ricin A coding sequence cleaved from the 3' end of the ricin A fragment during the FspI digest, plus a 1/2 ApaI site for fusion to plasmid pPA123.

The completed construct is then cloned into NcoI/ApaI-digested pPA123 to yield a ricin A-diphtheria toxin B'-IL2 gene that can be expressed in E.coli from the tcr promoter on the plasmid (see Fig. 12).

Purified ricin A-diphtheria toxin B'-IL2 hybrid, like the SLTA/DTB'/IL2 hybrid of Example 3, inactivates ribosomes in cells bearing IL2 receptors, resulting in cessation of protein synthesis and death of the targeted cells. The ricin A hybrid would thus have the same applications as SLTA/DTB'/IL2 hybrid, as discussed in Example 3.

Example 5. Construction of phenylalanine hydroxylase- diphtheria toxin B gene, and use of the resultant hybrid protein.

A human liver cDNA library is screened for phenylalanine hydroxylase ("PH") cDNA as described by Kwok et al., Biochem. 24 :556-561, 1985. The approximately 1160-bp EcoRII-AflII fragment that encodes most of the PH protein is isolated (see Figs. 13 and 14). The following linkers are ligated onto the 5' EcoRII end in order to recreate the 5' coding sequences and incorporate an NcoI site :

```
      5'  - C ATG TCC ACT GCG GTC CTG GAA AAC        - 3'
      3'  -     AGG TGA CGC CAG GAC CTT TTG GGT CC  - 5'
          fmet-Ser-Thr-Ala-Val-Leu-Gln-Asn-Pro-Gly
          NcoI                                1/2
          sticky end                          EcoRII
```

The following linkers are ligated onto the 3' AflII end to complete the PH coding sequence and to include an ApaI restriction site in the correct translational reading frame for fusion to diphtheria toxin fragment B sequences (Fig. 14) :

```
  5' - TT AAG ATT TTG GCT GAT TCC ATT AAC AGT GAA ATT GGA-
  3' -    C TAA AAC CGA CTA AGG TAA TTG TCA CTT TAA CCT-
       Lys-Ile-Leu-Ala-Asp-Ser-Ile-Asn-Ser-Glu-Ile-Gly-
    1/2
    AflII
```

```
(cont.) -ATC CTT TGC AGT GCC CTC CAG AAA ATA AAG GGG CC - 3'
        -TAG GAA ACG TCA CGG GAG GTC TTT TAT TTC C      - 5'
        -Ile-Leu-Cys-Ser-Ala-Leu-Gln-Lys-Ile-Lys-Gly-Pro
                                                  1/2
                                                  ApaI
```

This fragment is then ligated onto the NcoI-ApaI digested pPA123 vector (Fig. 14) resulting in a plasmid that encodes phenylalanine hydroxylase fused to diphtheria toxin B'-IL-2. Finally, this plasmid is digested with EcoRI and SphI to remove IL-2 encoding sequences, which are replaced by the approximately 230 bp SphI-EcoRI fragment of corynebacteriophage ß that encodes the 3' end of diphtheria toxin fragment B (Fig. 14). This completed construct codes for a PH-diphtheria toxin B hybrid protein that can be expressed in E.coli from the trc promoter on the plasmid (see Fig. 14).

The inherited disorder phenylketonuria, in which the inability to metabolize phenylalanine leads to an

accumulation of excess phenylalanine and possible brain damage in affected individuals, has been attributed to a genetic deficiency of the enzyme PH. By constructing a molecule in which active PH enzyme is linked to the cell-binding and translocation domains of diphtheria toxin Fragment B, the enzyme can be targeted to and incorporated into the broad range of cells which native diphtheria toxin normally attacks, achieving the widespread therapy that is called for by a defect such as phenylketonuria. This cloning strategy would be applicable to the construction of other hybrids useful in the treatment of other genetic defects.

Example 6. Construction of an HIV protease-binding protein-diphtheria toxin B'-IL2 gene, and use of the resultant hybrid protein.

A recombinant gene expressing a novel protein, an antigen-binding, single-polypeptide-chain analog of a monoclonal antibody composed of an antibody variable light-chain amino acid sequence (V_L) linked to a variable heavy-chain sequence (V_H) by a linker peptide, is constructed by the method of Bird et al., Science 242 :423-426, 1988, based upon the V_L and V_H sequences of a monoclonal antibody specific for and able to inactivate HIV protease (Hansen et al., Embo J. 7 :1785-1791, 1988) and a linker peptide designed by the method of Bird et al. The ends of the V_L-linker-V_H gene are modified with appropriate restriction enzymes and synthetic DNA linkers in order to produce an intact V_L-linker-V_H gene having 1/2 of a NcoI site at the 5' end and 1/2 of an ApaI site at the 3' end. The gene is then cloned into NcoI + ApaI-digested pPA123 to produce a plasmid expressing, from the trc promoter, an HIV protease-binding protein-diphtheria toxin B'-IL2 hybrid protein ("HIVP-BP/DTB'/IL2 hybrid").

Following expression of the recombinant gene in E.coli, the HIVP-BP/DTB'/IL2 hybrid protein can be isolated and used to treat an HIV infection in a human patient. The HIV virus infects and proliferates within T-cells, commandeering the cellular protein synthesis machinery to produce multiple copies of its own proteins. One viral protein in particular, the HIV protease, plays a critical role in the processing of other viral proteins ; identifying a way to inactivate this protease within the infected cell has been the focus of much recent effort toward developing an effective AIDS therapy (see, e.g., Hansen et al.). The HIVP-BP/DTB'/IL2 hybrid delivers a viral protease-specific inhibitor specifically to activated T-cells bearing the IL2 receptor, and thus can be effective at low dosages, with little or no toxicity to other types of cells. This technology could be applied as well to other viral infections or genetic disorders.

Other Embodiments

Other embodiments are within the following claims. For example, any cell-specific polypeptide ligand can be used which has a binding domain specific for the particular class of cells which are to be labeled. Polypeptide hormones are useful such ligands. Hybrid protein made using the binding domain of α or ß MSH, for example, can selectively bind to melanocytes, rendering hybrids, once labelled with a detectable label, useful in the diagnosis of melanoma and the in vivo and in vitro detection of metastic melanoma loci. Such a hybrid, when attached to an enzymatically-active portion of a toxin molecule instead of to a detectable label, could be utilized to deliver that toxic activity specifically to the target melanoma cells. Other ligands provide different specificities: e.g., the binding domain of substance P recognizes receptors on the surfaces of neurons involved in the transmission of pain, so that labeled hybrids made using substance P can be used to map areas of the nervous system containing substance P receptors. Other specific-binding ligands which can be used include insulin, somatostatin, EGF, and Interleukins I, II, III, IV and VI. Interleukin II is of particular importance because of its role in allergic reactions and autoimmune diseases such as Systemic Lupus Erythmatosis (SLE), involving activated T cells. Other useful polypeptide ligands having cell-specific binding domains are follicle stimulating hormone (specific for ovarian cells), luteinizing hormone (specific for ovarian cells), thyroid stimulating hormone (specific for thyroid cells), vasopressin (specific for uterine cells, as well as bladder and intestinal cells), prolactin (specific for breast cells), and growth hormone (specific for certain bone cells). Alternatively, a relatively indiscriminate cell-binding ligand (such as that of diphtheria toxin or ricin toxin) capable of binding to a wide variety of cell types in an organism can be used to effect widespread introduction of a specific chemical entity into cells of that organism, where more specific targeting is not the goal.

For a number of cell-specific ligands, the region within each such ligand in which the binding domain is located is now known. Furthermore, recent advances in solid phase polypeptide synthesis enable those skilled in this technology to determine the binding domain of practically any such ligand, by synthesizing various fragments of the ligand and testing them for the ability to bind to the class of cells to be labeled. Thus, the hybrid molecules of the invention need not include an entire ligand, but rather may include only a fragment of a ligand which exhibits the desired cell-binding capacity. Likewise, analogs of the ligand or its cell-binding region having minor sequence variations may be synthesized, tested for their ability to bind to cells, and incorporated into

the hybrid molecules of the invention. Other potential ligands include monoclonal antibodies or antigen-binding, single-chain analogs of monoclonal antibodies, where the antigen is a receptor or other moiety expressed on the surface of the target cell membrane.

The translocation function of the hybrid molecule may be supplied by an appropriate piece of a polypeptide other than diphtheria toxin, but which is capable of translocating in a manner analogous to that of diphtheria toxin (e.g., Pseudomonas exotoxin A, botulinum, neurotoxin, or ricin), or in any other manner which accomplishes the objective of translocating the functional "third part" of the hybrid molecule into the cell's cytoplasm.

The chemical entity to be inserted into the cell can vary widely and still be within the invention. For example, the enzyme which is genetically deficient in Tay-Sachs disease, hexosaminidase A, could be attached to a hybrid having a cell-binding domain specific for the cells most affected by the disease, nerve cells. Patients suffering from type 2 glycogenosis might be treated with a hybrid comprising α-1,4-glucosidase linked to the translocation segment of diphtheria toxin linked to insulin, which would largely target muscle cells, hepatocytes, and lymphocytes. (See Poznansky et al., Science 223 :1304-1306, 1984.) These are simply examples : any other enzyme deficiency disease for which the natural enzyme or its gene has been sequenced (or is amenable to sequencing by one skilled in the art, without undue experimentation) could be treated with a hybrid comprising the active enzyme linked to a translocation domain linked to an appropriate cell-binding ligand.

Intracellular viral and bacterial infections could be treated by an appropriate hybrid : for example, a hybrid which delivers into the cell a potent antibiotic, or a recombinant V_L-linker-V_H antigen-binding polypeptide which specifically binds viral particles or proteins.

Likewise, the hybrid of the invention will be useful for specifically destroying certain cells. Besides the cholera toxin A_1-hybrid, ricin A-hybrid and Shiga-like toxin A-hybrid exemplified above, a cell-killing function may be provided by the enzymatically-active portion of any polypeptide toxin, including but not limited to LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin and Pseudomonas exotoxin A. Cells to be targeted might include cancer cells, virus-infected cells, or adipocytes.

The invention includes biologically active mutational analogs of hybrid polypeptides described above. By manipulating the recombinant DNA sequence encoding the subject hybrid polypeptide using methods well known to those of ordinary skill in the art of genetic engineering, a series of mutations involving deletions and/or substitutions of individual or multiple base pairs in such recombinant DNA sequence is first created. Each such mutated sequence is then inserted into an expression vector and expressed in an appropriate expression system. The biological activity of the mutational analog so produced can then be compared to that exhibited by the hybrid molecule of which it is an analog (the "parent polypeptide"). The particular assay used will depend upon the particular enzymatic activity and cell-binding specificity of the parent polypeptide. For example, mutational analogs of the Shiga-like toxin A/diphtheria toxin B'/IL2 (SLTA/DTB'/IL2) hybrid, the cholera toxin A_1/diphtheria toxin B'/IL2 (CTA/DTB'/IL2) hybrid, and the ricin A/ diphtheria toxin B'/IL2 hybrid may be tested and compared to their respective parent polypeptides in the following cell cytotoxicity assay, which is specific for toxins capable of binding to IL2 receptor-bearing cells.

Assay

Cultured HUT 102/6TG (Tsudo et al., Proc. Natl. Acad. Sci. USA 83 :9694, 1986) or YT2C2 (Teshigawari et al., J. Exp. Med. 165 :223, 1987) cells are maintained in RPMI 1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Cellect, GIBCO), 2mM glutamine, and penicillin and streptomycin to 50 IU and 50 µg/ml, respectively. Cells are seeded in 96-well V-bottomed plates (Linbro-Flow Laboratories, McLean, VA) at a concentration of 5×10^4 per well in complete medium. Putative toxins are added to varying concentrations (10^{-12}M to 10^{-6}M) and the cultures are incubated for 18 hrs. at 37°C in a 5% CO_2 atmosphere. Following incubation, the plates are centrifuged for 5 min. at 170 x g, and the medium removed and replaced with 200 µl leucine-free medium (MEM, Gibco) containing 1.0 µCi/ml [^{14}C]-leucine (New England Nuclear, Boston, MA). After an additional 90 min. at 37°C, the plates are centrifuged for 5 min. at 170 x g, the medium is removed and the cells are lysed by the addition of 4 M KOH. Protein is precipitated by the addition of 10% trichloroacetic acid and the insoluble material is then collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters are washed, dried, and counted according to standard methods. Cells cultured with medium alone serve as the control.

Where IL4 replaces IL2 as the cell-binding portion of the resulting hybrid, the hybrid and its mutational analogs may be tested by a similar assay utilizing CT4R cells (William E. Paul, NIH), P815 cells (ATCC), or CTLL2 (ATCC), seeded at 1×10^4 cells per well and incubated for 40 hours.

Claims

1. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,
(a) said first part comprising a portion of the binding domain of a cell-binding ligand, said portion being effective to cause said hybrid molecule to bind to a cell of an animal ;
(b) said second part comprising a portion of a translocation domain of a protein capable of translocating said third part across the cytoplasmic membrane of said cell ; and
(c) said third part comprising a chemical entity to be introduced into said cell, provided that (i) said hybrid molecule is produced by expression of a recombinant DNA molecule encoding said hybrid molecule, and (ii) said second part and said third part are not segments of the same naturally-occurring polypeptide toxin.

2. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,
(a) said first part comprising a portion of the binding domain of a cell-binding ligand, said portion being effective to cause said hybrid molecule to bind to a cell of an animal ;
(b) said second part comprising a portion of a translocation domain of a protein capable of translocating said third part across the cytoplasmic membrane of said cell ; and
(c) said third part comprising a chemical entity to be introduced into said cell, provided that (i) said first part and said second part are not segments of the same naturally-occurring polypeptide toxin, and (ii) said second part and said third part are not segments of the same naturally-occurring polypeptide toxin.

3. The hybrid molecule of claim 1 or claim 2, wherein said protein is a naturally-occurring toxin.

4. The hybrid molecule of claim 2, wherein said protein is selected from the group consisting of diphtheria toxin and Pseudomonas exotoxin A.

5. The hybrid molecule of claim 1 or claim 2, wherein said ligand is a polypeptide hormone.

6. The hybrid molecule of claim 5, wherein said hormone is selected from the group consisting of insulin, Interleukin II, Interleukin IV, Interleukin VI, and EGF.

7. The hybrid molecule of claim 2, wherein said ligand is a steroid hormone.

8. The hybrid molecule of claim 1 or claim 2, wherein said ligand is an antigen-binding, single-chain analog of a monoclonal antibody, and said antigen is expressed on the surface of said cell.

9. The hybrid molecule of claim 1 or claim 2, wherein said ligand is a polypeptide toxin capable of binding to said cell.

10. The hybrid molecule of claim 2, wherein any adjacent two of said parts are both polypeptides, and said covalent bond between said two parts is a peptide bond.

11. The hybrid molecule of claim 10, wherein said first part and said third part each comprises a polypeptide and said hybrid molecule comprises a recombinant protein.

12. The hybrid molecule of claim 1 or claim 2, wherein said chemical entity comprises an enzymatically-active portion of an enzyme.

13. The hybrid molecule of claim 12, wherein said enzyme is a toxin.

14. The hybrid molecule of claim 13, wherein said enzyme is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin and Pseudomonas exotoxin A.

15. The hybrid molecule of claim 14, wherein said enzyme is cholera toxin.

16. The hybrid molecule of claim 12, wherein said enzyme is selected from the group consisting of hexosaminidase A, α-1,4-glucosidase, phenylalanine hydroxylase, a protease and a nuclease.

17. The hybrid molecule of claim 12, wherein said enzymatically-active portion supplies an enzymatic activity in which said cell is deficient.

18. The hybrid molecule of claim 17, wherein said deficiency is genetic.

19. The hybrid molecule of claim 1 or claim 2, wherein said chemical entity comprises an antigen- binding, single-chain analog of a monoclonal antibody.

20. The hybrid molecule of claim 19, wherein said antigen is a viral protein.

21. The hybrid molecule of claim 20, wherein said viral protein is HIV protease.

22. A hybrid polypeptide molecule comprising CTA/DTB'/IL2 hybrid.

23. A hybrid polypeptide molecule comprising SLTA/DTB'/IL2 hybrid.

24. A hybrid polypeptide molecule comprising ricin A-diphtheria toxin B'-IL2 hybrid.

25. A hybrid polypeptide molecule encoded by the phenylalanine hydroxylase-diphtheria toxin fragment B gene of Fig. 14.

26. A hybrid polypeptide molecule comprising HIVP-BP/DTB'/IL2 hybrid.

27. A hybrid polypeptide molecule comprising a biologically active mutational analog of the hybrid molecule of any of claims 22-26.

28. A hybrid polypeptide molecule comprising an enzymatically-active portion of Shiga-like toxin A and a portion of a binding domain of a cell-binding polypeptide ligand, said portion of said binding domain being effective to cause said hybrid molecule to bind to a cell of an animal.

29. The hybrid molecule of claim 28, wherein said polypeptide ligand is IL2.

30. A method of treating an animal having a disease characterized by the enzyme deficiency of claim 17, said method comprising administering to said animal an effective amount of the hybrid molecule of claim 17.

31. A method of raising the cyclic AMP level within a target cell, said method comprising contacting said target cell with the hybrid molecule of claim 15.

32. The method of claim 31, wherein said target cell is a T cell and said hybrid molecule comprises at least a portion of the binding domain of Interleukin II.

33. A method of treating a human patient infected with HIV, said method comprising administering to said patient an effective amount of the hybrid molecule of claim 21.

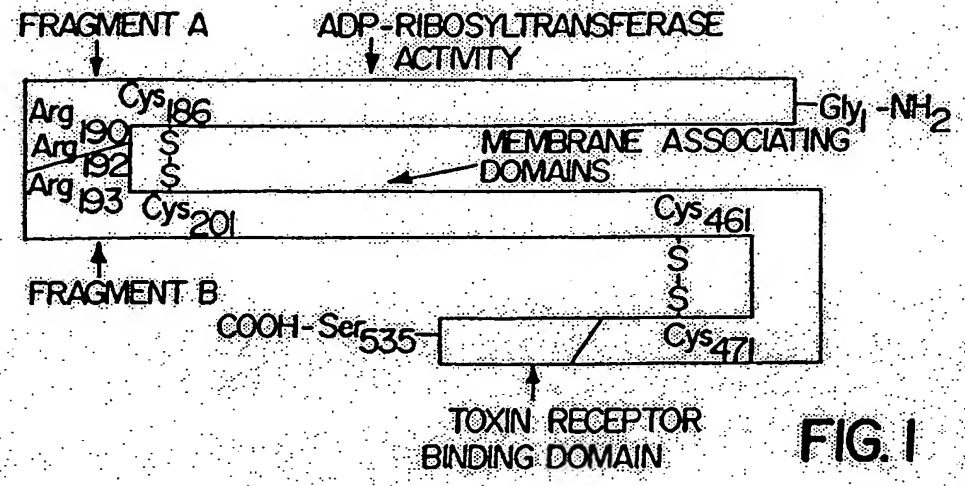
34. A recombinant DNA molecule encoding the hybrid protein of claim 1 or claim 2.

35. A vector comprising the recombinant DNA molecule of claim 34.

36. A cell containing the recombinant DNA molecule of claim 34.

37. The cell of claim 36, wherein said cell is capable of expressing said recombinant DNA molecule.

38. A method of preparing the hybrid molecule of claim 1 or claim 2, said method comprising
providing a cell containing a recombinant DNA molecule encoding said hybrid molecule (the "transformed cell"), and
permitting said transformed cell to express said recombinant DNA molecule.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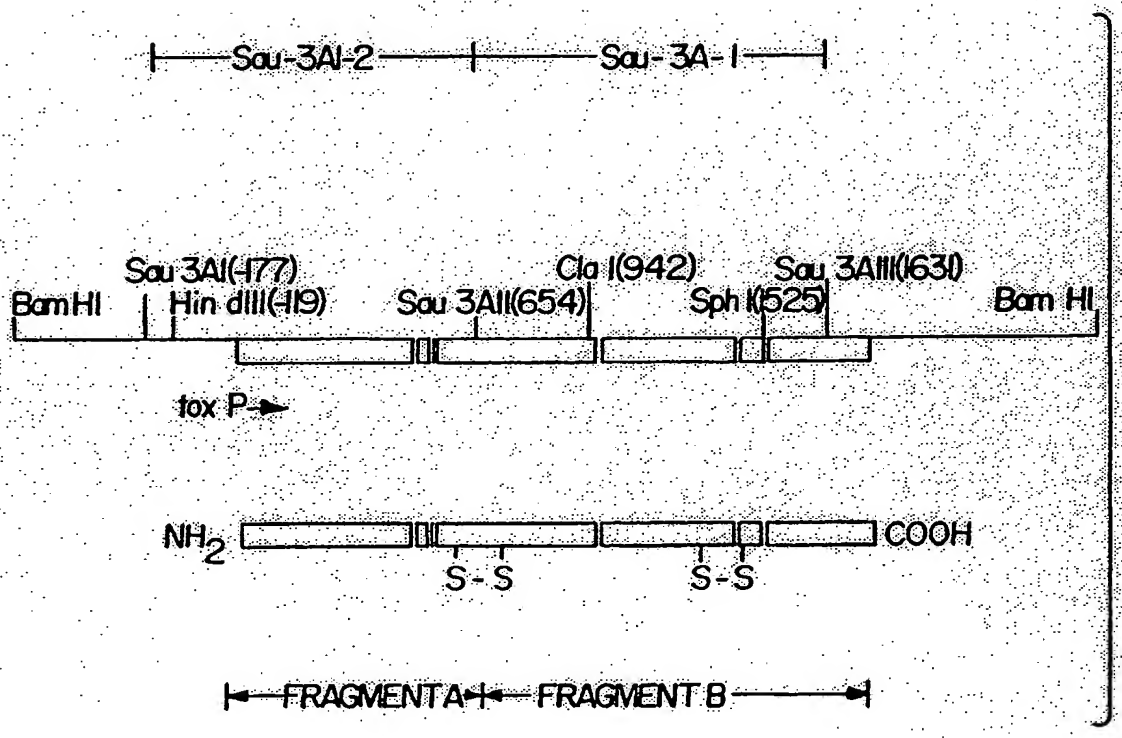
Arg 190
Arg 192
Arg 193
Cys 186
Cys 201
Gly 1 -NH2
MEMBRANE ASSOCIATING DOMAINS
Cys 461
Cys 471
FRAGMENT B
COOH-Ser 535
TOXIN RECEPTOR BINDING DOMAIN

FIG. 1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Sau-3A-1
Sau 3AI(-177)
Bam HI
Hin dIII(-19)
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Cla I(942)
Sph I(525)
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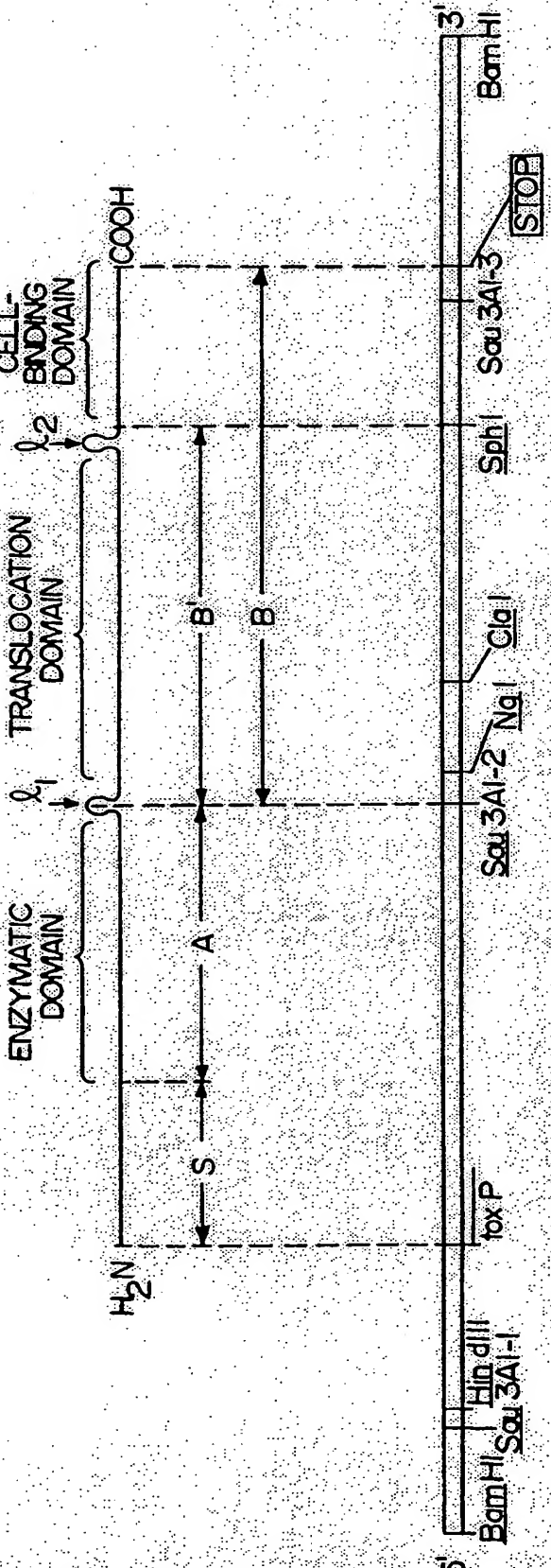
Bam HI
tox P
NH2
COOH
S-S
S-S
FRAGMENT A
FRAGMENT B

FIG. 2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CELL-BINDING DOMAIN
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ℓ_2
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B
5'
3'
BamHI
Sau3AI-1
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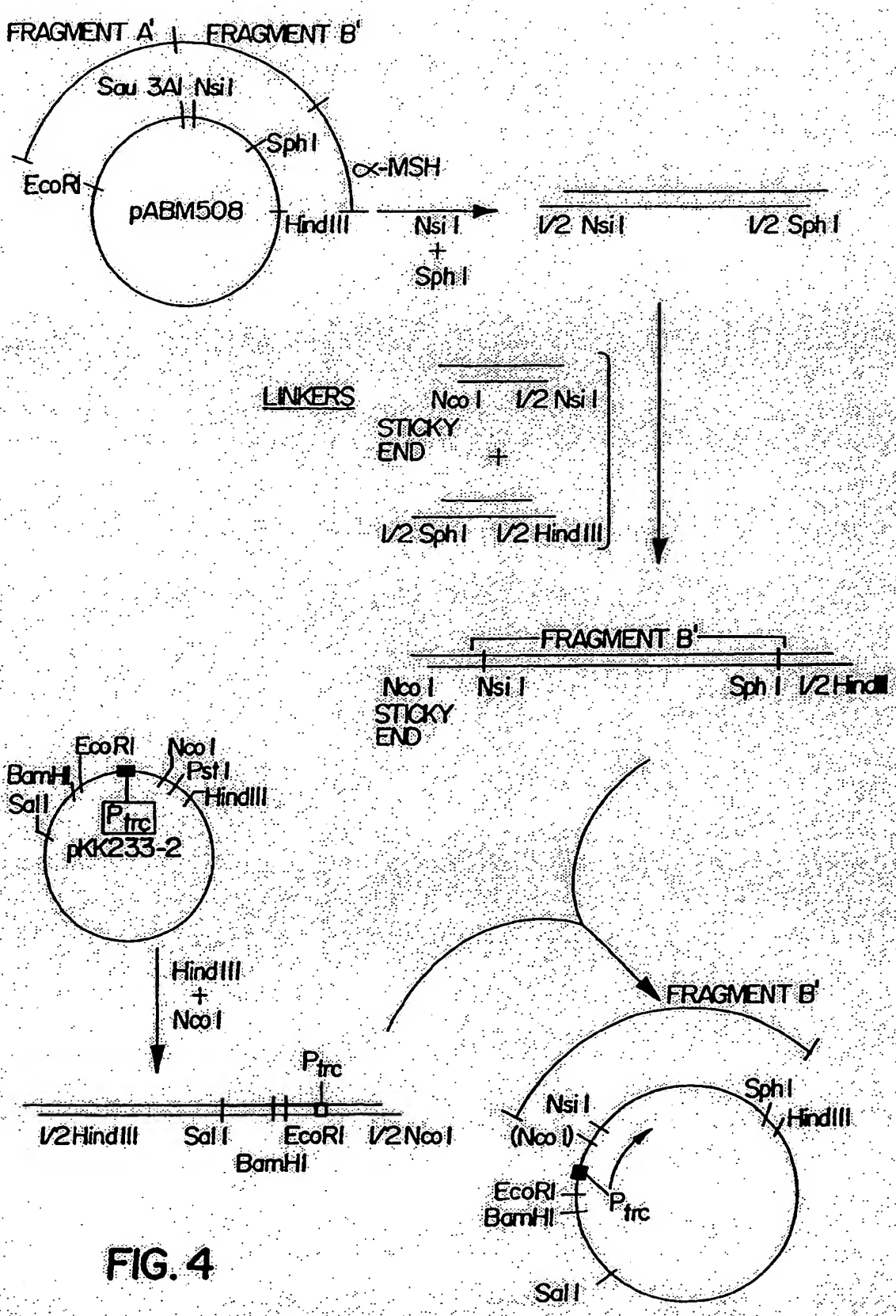
ClaI
SphI
Sau3AI-3
STOP
BamHI

FIG. 3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FRAGMENT B'
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+
SphI
1/2 NsiI
1/2 SphI
LINKERS
NcoI
1/2 NsiI
STICKY
END
+
1/2 SphI
1/2 HindIII
FRAGMENT B'
NcoI
NsiI
SphI
1/2 HindII
STICKY
END
EcoRI
NcoI
BamHI
PstI
SalI
HindIII
Ptrc
pKK233-2
HindIII
+
NcoI
Ptrc
1/2 HindIII
SalI
EcoRI
1/2 NcoI
BamHI
FRAGMENT B'
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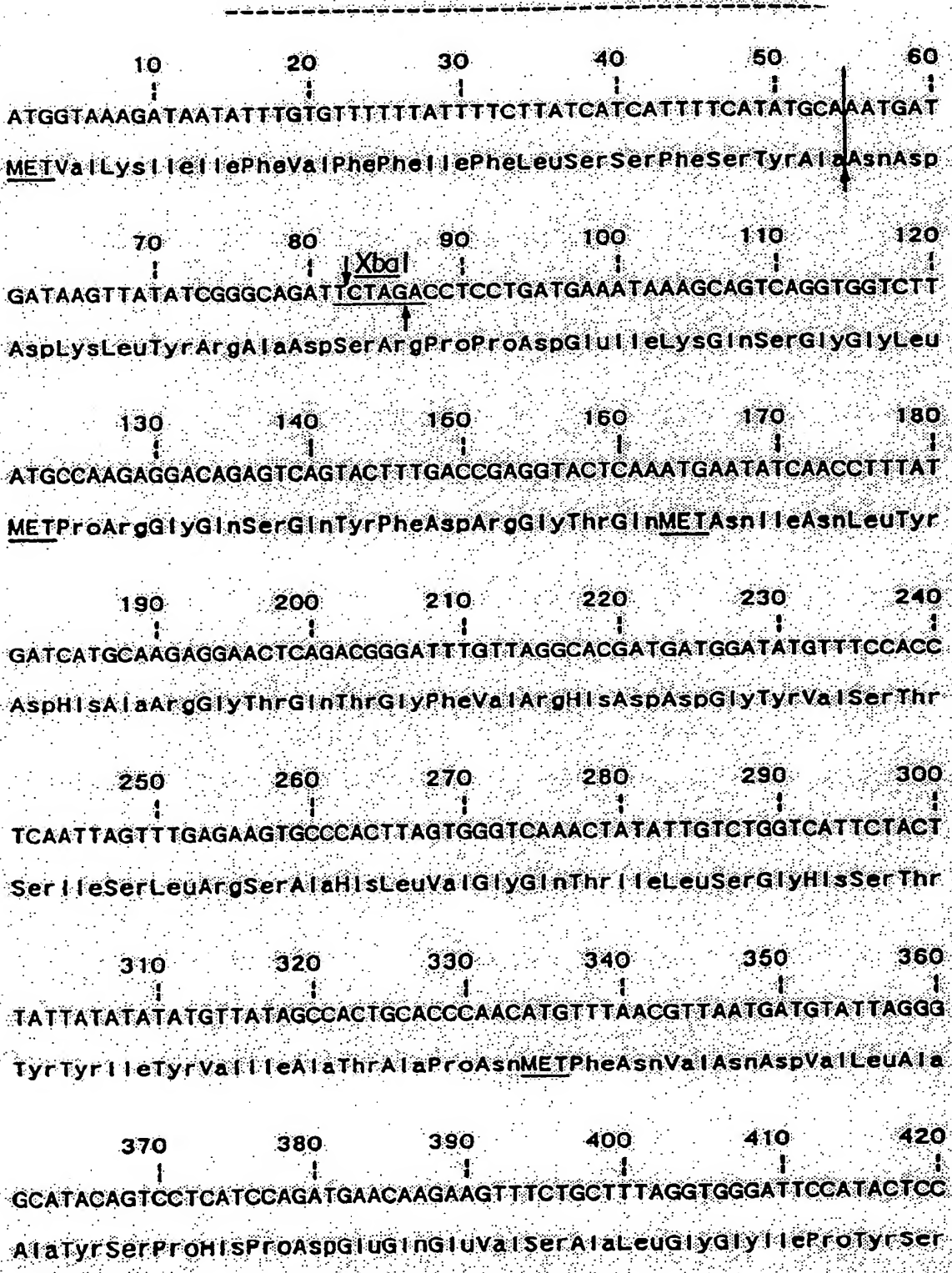
(NcoI)
HindIII
EcoRI
BamHI
Ptrc
SalI

FIG. 4

FIG. 5-1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70 80 XbaI 90 100 110 120
GATAAGTTATATCGGGCAGATTCTAGACCTCCTGATGAAATAAAGCAGTCAGGTGGTCTT
AspLysLeuTyrArgAlaAspSerArgProProAspGluIleLysGlnSerGlyGlyLeu
130 140 150 160 170 180
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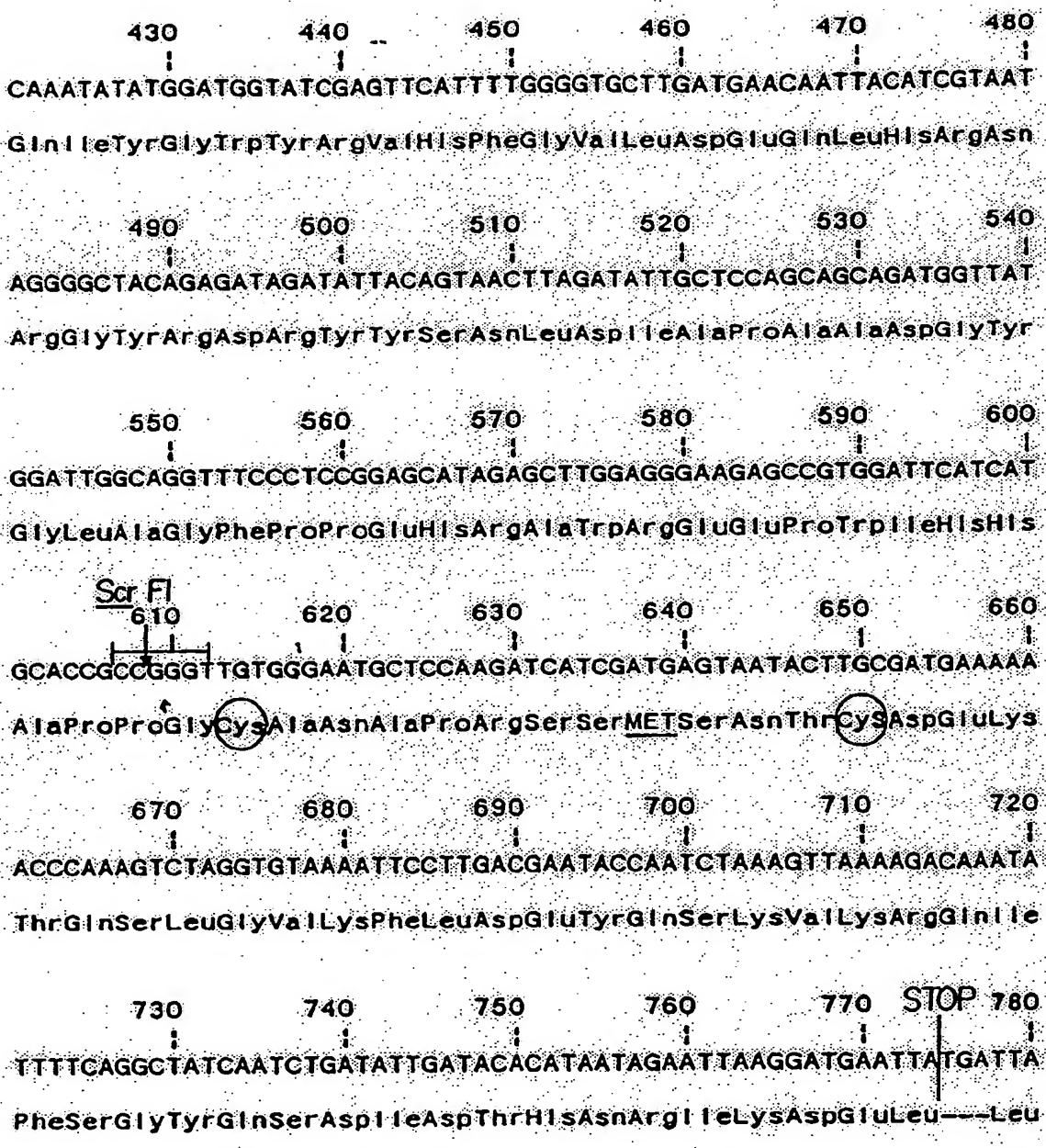
METProArgGlyGlnSerGlnTyrPheAspArgGlyThrGlnMETAsnIleAsnLeuTyr
190 200 210 220 230 240
GATCATGCAAGAGGAACTCAGACGGGATTTGTTAGGCACGATGATGGATATGTTTCCACC
AspHisAlaArgGlyThrGlnThrGlyPheValArgHisAspAspGlyTyrValSerThr
250 260 270 280 290 300
TCAATTAGTTTGAGAAGTGCCCACTTAGTGGGTCAAACTATATTGTCTGGTCATTCTACT
SerIleSerLeuArgSerAlaHisLeuValGlyGlnThrIleLeuSerGlyHisSerThr
310 320 330 340 350 360
TATTATATATATGTTATAGCCACTGCACCCAACATGTTTAACGTTAATGATGTATTAGGG
TyrTyrIleTyrValIleAlaThrAlaProAsnMETPheAsnValAsnAspValLeuAla
370 380 390 400 410 420
GCATACAGTCCTCATCCAGATGAACAAGAAGTTTCTGCTTTAGGTGGGATTCCATACTCC
AlaTyrSerProHisProAspGluGlnGluValSerAlaLeuGlyGlyIleProTyrSer

FIG. 5-2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490 500 510 520 530 540
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ArgGlyTyrArgAspArgTyrTyrSerAsnLeuAspIleAlaProAlaAlaAspGlyTyr
550 560 570 580 590 600
GGATTGGCAGGTTTCCCTCCGGAGCATAGAGCTTGGAGGGAAGAGCCGTGGATTCATCAT
GlyLeuAlaGlyPheProProGluHisArgAlaTrpArgGluGluProTrpIleHisHis
Scr FI
610 620 630 640 650 660
GCACCGCCGGGTTGTGGGAATGCTCCAAGATCATCGATGAGTAATACTTGCGATGAAAAA
AlaProProGlyCysAlaAsnAlaProArgSerSerMETSerAsnThrCysAspGluLys
670 680 690 700 710 720
ACCCAAAGTCTAGGTGTAAAATTCCTTGACGAATACCAATCTAAAGTTAAAAGACAAATA
ThrGlnSerLeuGlyValLysPheLeuAspGluTyrGlnSerLysValLysArgGlnIle
730 740 750 760 770 STOP 780
TTTTCAGGCTATCAATCTGATATTGATACACATAATAGAATTAAGGATGAATTATGATTA
PheSerGlyTyrGlnSerAspIleAspThrHisAsnArgIleLysAspGluLeu---Leu

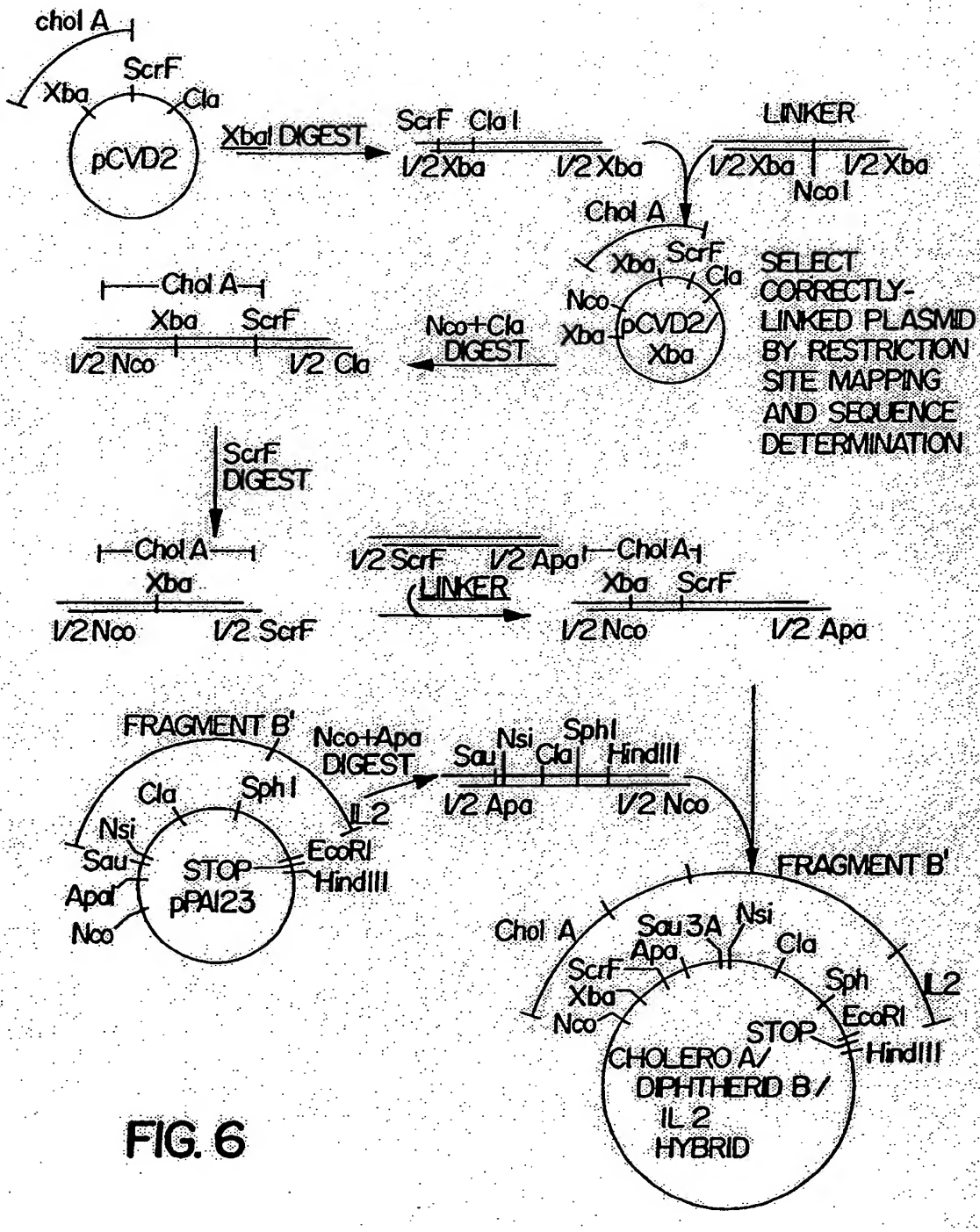

chol A
ScrF
Xba
Cla
pCVD2
XbaI DIGEST
ScrF Cla I
1/2 Xba
1/2 Xba
LINKER
1/2 Xba
1/2 Xba
Nco I
Chol A
Xba
ScrF
Cla
Nco
Xba
pCVD2/
Xba
SELECT CORRECTLY-LINKED PLASMID BY RESTRICTION SITE MAPPING AND SEQUENCE DETERMINATION
Nco+Cla DIGEST
Chol A
Xba
ScrF
1/2 Nco
1/2 Cla
ScrF DIGEST
Chol A
Xba
1/2 Nco
1/2 ScrF
1/2 ScrF
1/2 Apa
LINKER
Chol A
Xba
ScrF
1/2 Nco
1/2 Apa
FRAGMENT B'
Nco+Apa DIGEST
Cla
Sph I
Nsi
Sau
Apa I
Nco
STOP
pPAI23
IL 2
EcoRI
HindIII
Nsi
Sph I
Sau
Cla
HindIII
1/2 Apa
1/2 Nco
FRAGMENT B'
Chol A
Sau 3A
Nsi
Apa
Cla
ScrF
Xba
Nco
Sph
IL2
EcoRI
STOP
HindIII
CHOLERO A/
DIPHTHERID B/
IL 2
HYBRID

FIG. 6

FIG. 7

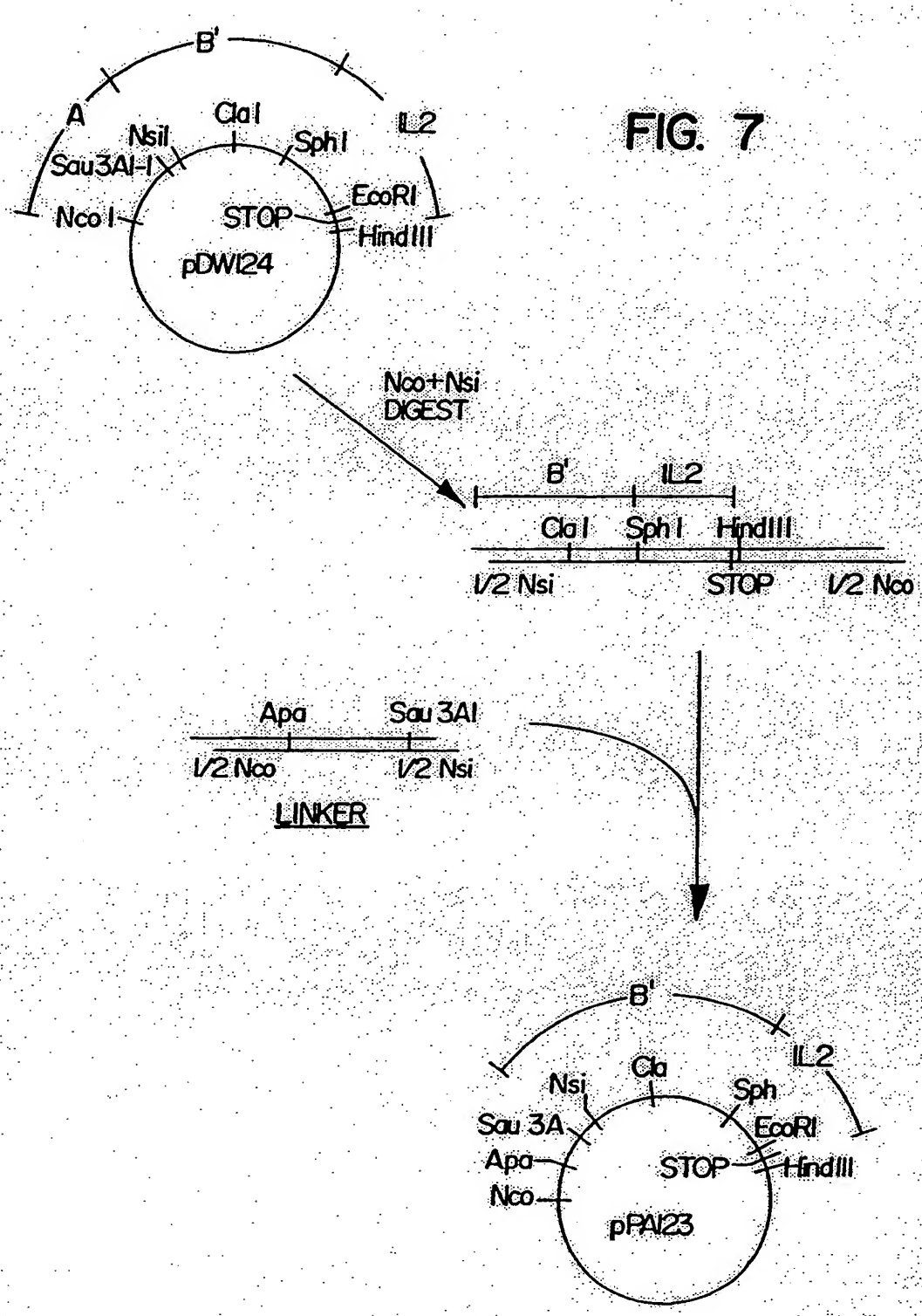

B'
A
NsiI
Sau3AI-I
ClaI
SphI
IL2
NcoI
STOP
EcoRI
HindIII
pDW124
Nco+Nsi
DIGEST
B'
IL2
ClaI
SphI
HindIII
1/2 Nsi
STOP
1/2 Nco
Apa
Sau 3AI
1/2 Nco
1/2 Nsi
LINKER
B'
IL2
Nsi
Cla
Sph
Sau 3A
EcoRI
Apa
STOP
HindIII
Nco
pPAI23

FIG. 8-1

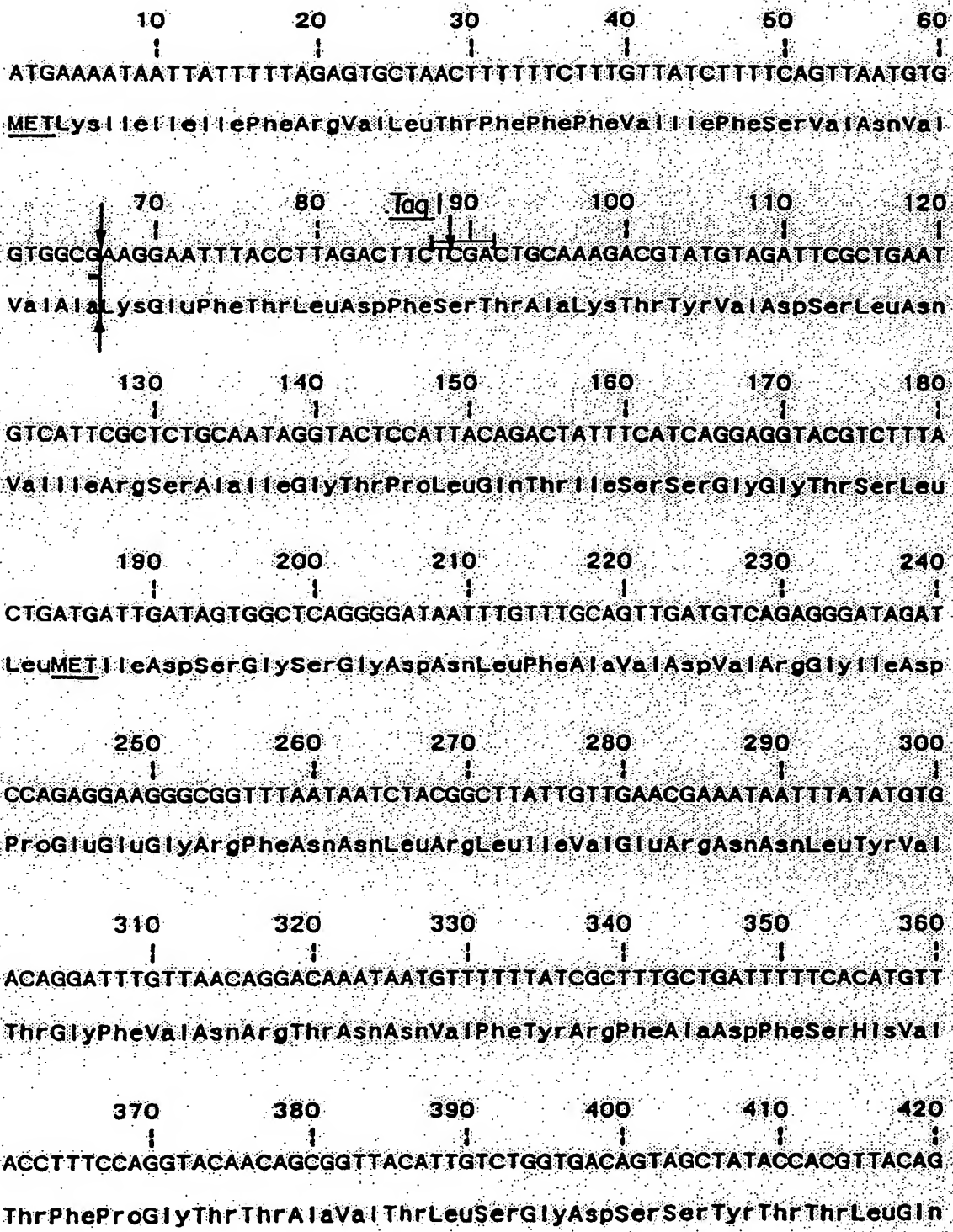

10 20 30 40 50 60
ATGAAAATAATTATTTTTAGAGTGCTAACTTTTTTCTTTGTTATCTTTTCAGTTAATGTG
METLysIleIleIlePheArgValLeuThrPhePhePheValIlePheSerValAsnVal
70 80 TaqI 90 100 110 120
GTGGCGAAGGAATTTACCTTAGACTTCTCGACTGCAAAGACGTATGTAGATTCGCTGAAT
ValAlaLysGluPheThrLeuAspPheSerThrAlaLysThrTyrValAspSerLeuAsn
130 140 150 160 170 180
GTCATTCGCTCTGCAATAGGTACTCCATTACAGACTATTTCATCAGGAGGTACGTCTTTA
ValIleArgSerAlaIleGlyThrProLeuGlnThrIleSerSerGlyGlyThrSerLeu
190 200 210 220 230 240
CTGATGATTGATAGTGGCTCAGGGGATAATTTGTTTGCAGTTGATGTCAGAGGGATAGAT
LeuMETIleAspSerGlySerGlyAspAsnLeuPheAlaValAspValArgGlyIleAsp
250 260 270 280 290 300
CCAGAGGAAGGGCGGTTTAATAATCTACGGCTTATTGTTGAACGAAATAATTTATATGTG
ProGluGluGlyArgPheAsnAsnLeuArgLeuIleValGluArgAsnAsnLeuTyrVal
310 320 330 340 350 360
ACAGGATTTGTTAACAGGACAAATAATGTTTTTTATCGCTTTGCTGATTTTTCACATGTT
ThrGlyPheValAsnArgThrAsnAsnValPheTyrArgPheAlaAspPheSerHisVal
370 380 390 400 410 420
ACCTTTCCAGGTACAACAGCGGTTACATTGTCTGGTGACAGTAGCTATACCACGTTACAG
ThrPheProGlyThrThrAlaValThrLeuSerGlyAspSerSerTyrThrThrLeuGln

FIG. 8-2

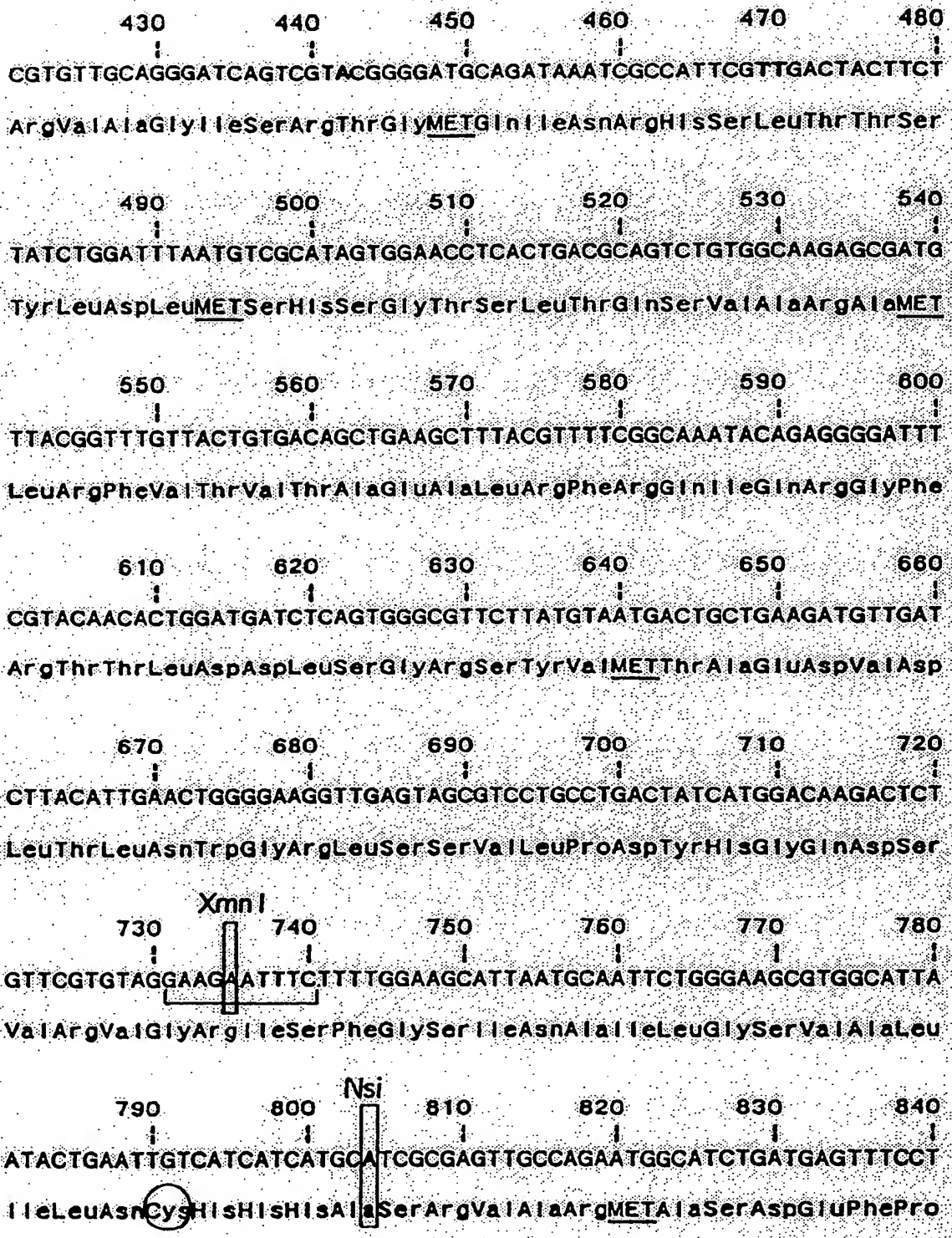
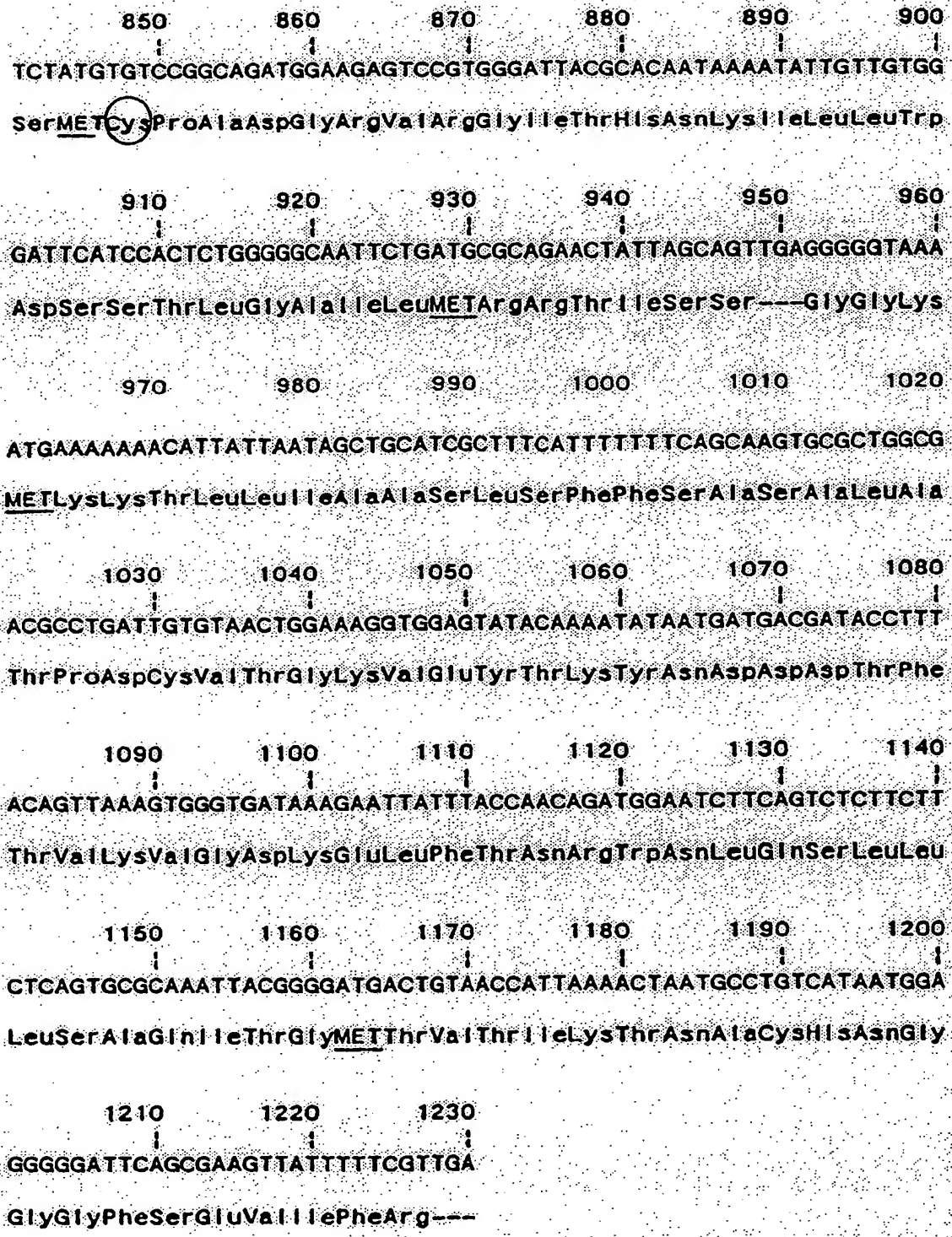

430 440 450 460 470 480
CGTGTTGCAGGGATCAGTCGTACGGGGATGCAGATAAATCGCCATTCGTTGACTACTTCT
ArgValAlaGlyIleSerArgThrGlyMETGlnIleAsnArgHisSerLeuThrThrSer
490 500 510 520 530 540
TATCTGGATTTAATGTCGCATAGTGGAACCTCACTGACGCAGTCTGTGGCAAGAGCGATG
TyrLeuAspLeuMETSerHisSerGlyThrSerLeuThrGlnSerValAlaArgAlaMET
550 560 570 580 590 600
TTACGGTTTGTTACTGTGACAGCTGAAGCTTTACGTTTTCGGCAAATACAGAGGGGATTT
LeuArgPheValThrValThrAlaGluAlaLeuArgPheArgGlnIleGlnArgGlyPhe
610 620 630 640 650 660
CGTACAACACTGGATGATCTCAGTGGGCGTTCTTATGTAATGACTGCTGAAGATGTTGAT
ArgThrThrLeuAspAspLeuSerGlyArgSerTyrValMETThrAlaGluAspValAsp
670 680 690 700 710 720
CTTACATTGAACTGGGGAAGGTTGAGTAGCGTCCTGCCTGACTATCATGGACAAGACTCT
LeuThrLeuAsnTrpGlyArgLeuSerSerValLeuProAspTyrHisGlyGlnAspSer
XmnI
730 740 750 760 770 780
GTTCGTGTAGGAAGAATTTCTTTTGGAAGCATTAATGCAATTCTGGGAAGCGTGGCATTA
ValArgValGlyArgIleSerPheGlySerIleAsnAlaIleLeuGlySerValAlaLeu
NsiI
790 800 810 820 830 840
ATACTGAATTGTCATCATCATGCATCGCGAGTTGCCAGAATGGCATCTGATGAGTTTCCT
IleLeuAsnCysHisHisHisAlaSerArgValAlaArgMETAlaSerAspGluPhePro

FIG. 8-3


850 860 870 880 890 900
TCTATGTGTCCGGCAGATGGAAGAGTCCGTGGGATTACGCACAATAAAATATTGTTGTGG
SerMETCysProAlaAspGlyArgValArgGlyIleThrHisAsnLysIleLeuLeuTrp
910 920 930 940 950 960
GATTCATCCACTCTGGGGGCAATTCTGATGCGCAGAACTATTAGCAGTTGAGGGGGTAAA
AspSerSerThrLeuGlyAlaIleLeuMETArgArgThrIleSerSer---GlyGlyLys
970 980 990 1000 1010 1020
ATGAAAAAAACATTATTAATAGCTGCATCGCTTTCATTTTTTTCAGCAAGTGCGCTGGCG
METLysLysThrLeuLeuIleAlaAlaSerLeuSerPhePheSerAlaSerAlaLeuAla
1030 1040 1050 1060 1070 1080
ACGCCTGATTGTGTAACTGGAAAGGTGGAGTATACAAAATATAATGATGACGATACCTTT
ThrProAspCysValThrGlyLysValGluTyrThrLysTyrAsnAspAspAspThrPhe
1090 1100 1110 1120 1130 1140
ACAGTTAAAGTGGGTGATAAAGAATTATTTACCAACAGATGGAATCTTCAGTCTCTTCTT
ThrValLysValGlyAspLysGluLeuPheThrAsnArgTrpAsnLeuGlnSerLeuLeu
1150 1160 1170 1180 1190 1200
CTCAGTGCGCAAATTACGGGGATGACTGTAACCATTAAAACTAATGCCTGTCATAATGGA
LeuSerAlaGlnIleThrGlyMETThrValThrIleLysThrAsnAlaCysHisAsnGly
1210 1220 1230
GGGGGATTCAGCGAAGTTATTTTTCGTTGA
GlyGlyPheSerGluValIlePheArg---

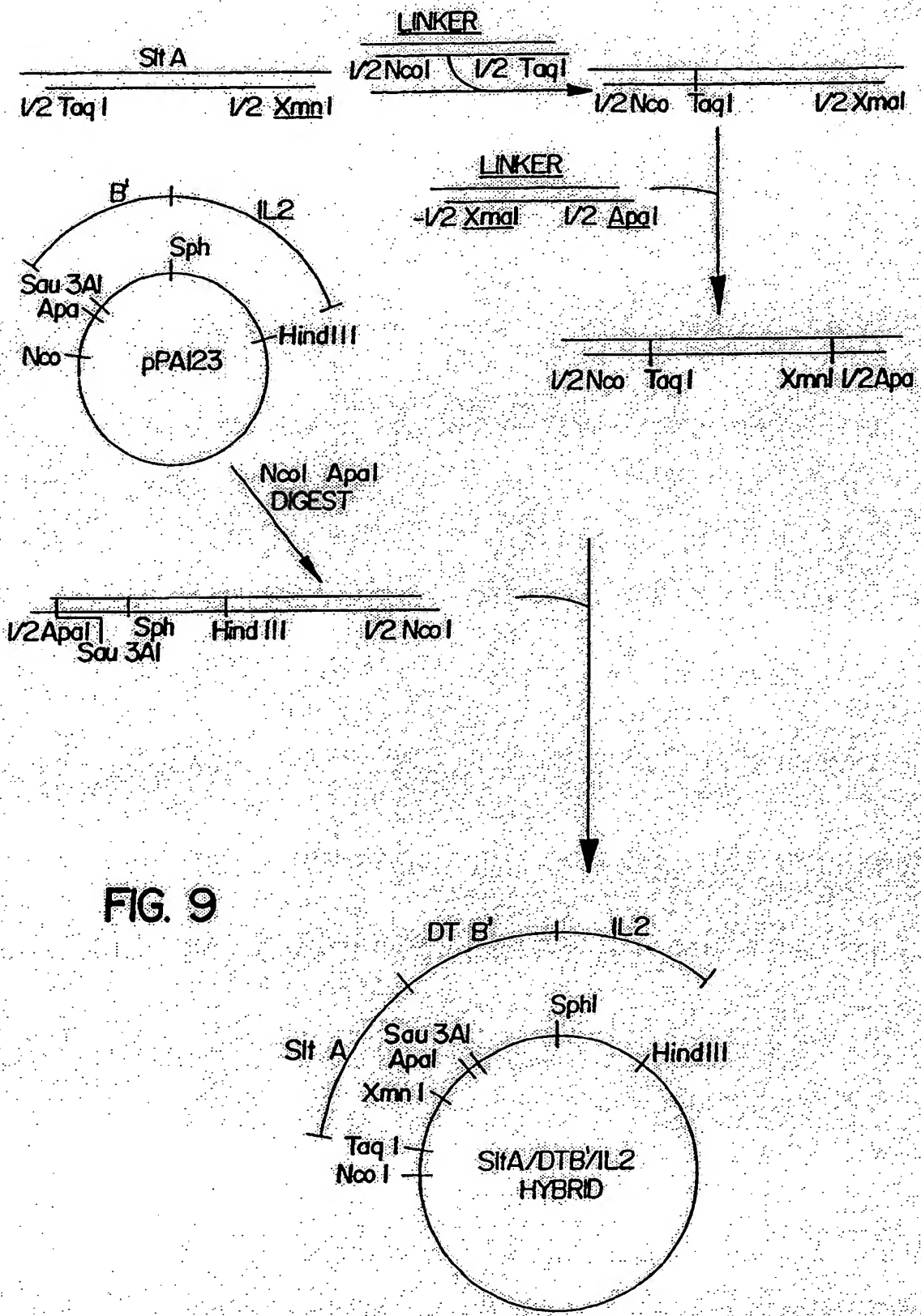

LINKER
Slt A
1/2 Nco I
1/2 Taq I
1/2 Taq I
1/2 Xmn I
1/2 Nco
Taq I
1/2 Xmal
LINKER
1/2 Xmal
1/2 Apa I
B'
IL2
Sph
Sau 3AI
Apa
Hind III
Nco
pPA123
1/2 Nco
Taq I
Xmn I
1/2 Apa
Nco I Apa I
DIGEST
1/2 Apa I
Sau 3AI
Sph
Hind III
1/2 Nco I
DT B'
IL2
Sph I
Slt A
Sau 3AI
Apa I
Hind III
Xmn I
Taq I
Nco I
Slt A/DTB'/IL2
HYBRID

FIG. 9

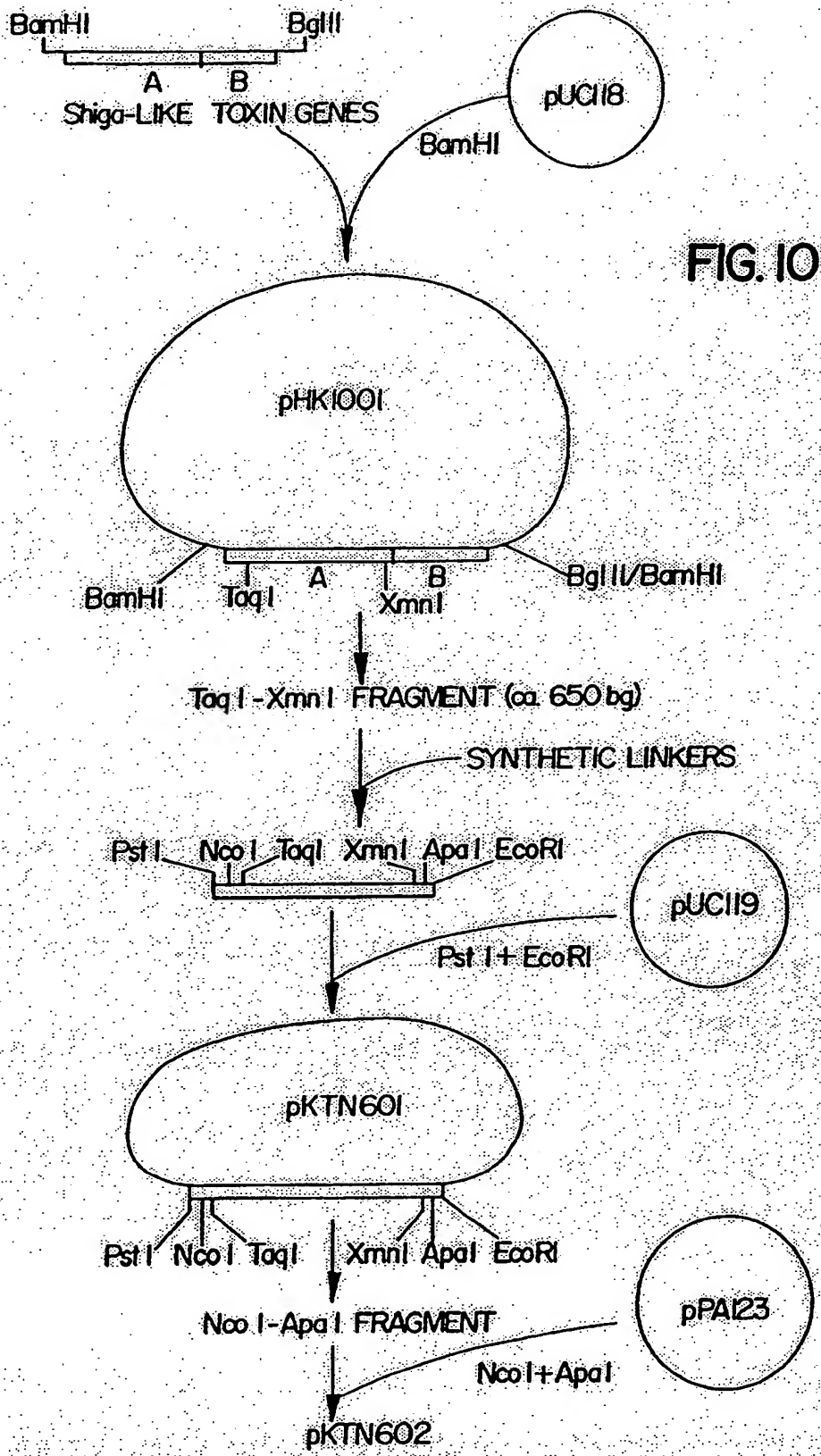

BamHI
BglII
A
B
Shiga-LIKE TOXIN GENES
pUC118
BamHI
pHK1001
BamHI
TaqI
A
B
XmnI
BglII/BamHI
TaqI-XmnI FRAGMENT (ca. 650 bg)
SYNTHETIC LINKERS
PstI
NcoI
TaqI
XmnI
ApaI
EcoRI
pUC119
PstI+EcoRI
pKTN601
PstI
NcoI
TaqI
XmnI
ApaI
EcoRI
NcoI-ApaI FRAGMENT
pPA123
NcoI+ApaI
pKTN602

FIG. 10

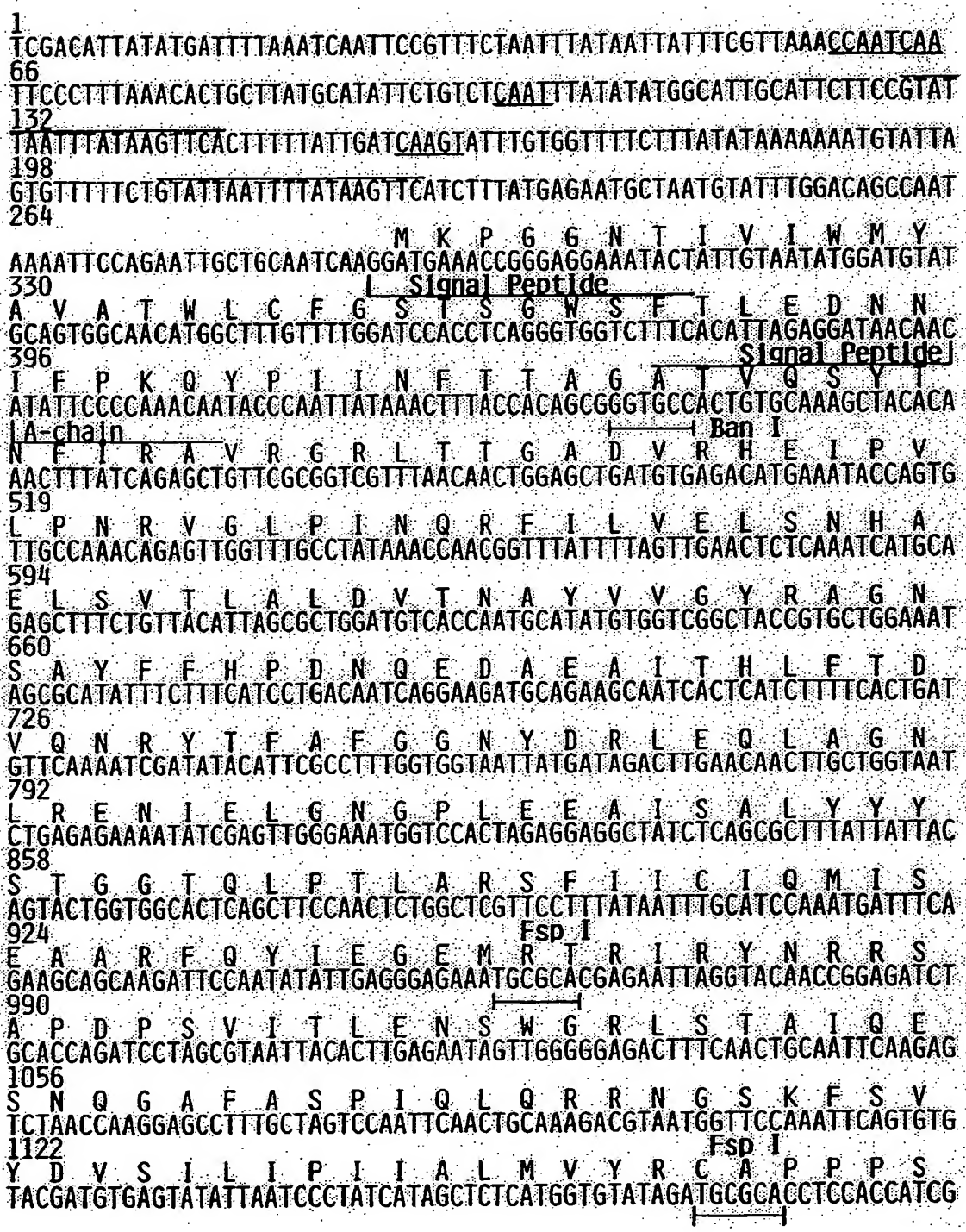

1
TCGACATTATATGATTTTAAATCAATTCCGTTTCTAATTTATAATTATTTCGTTAAACCAATCAA
66
TTCCCTTTAAACACTGCTTATGCATATTCTGTCTCAATTTATATATGGCATTGCATTCTTCCGTAT
132
TAATTTATAAGTTCACTTTTTATTGATCAAGTATTTGTGGTTTTCTTTATATAAAAAAATGTATTA
198
GTGTTTTTCTGTATTAATTTTATAAGTTCATCTTTATGAGAATGCTAATGTATTTGGACAGCCAAT
264
M K P G G N T I V I W M Y
AAAATTCCAGAATTGCTGCAATCAAGGATGAAACCGGGAGGAAATACTATTGTAATATGGATGTAT
330
Signal Peptide
A V A T W L C F G S T S G W S F T L E D N N
GCAGTGGCAACATGGCTTTGTTTTGGATCCACCTCAGGGTGGTCTTTCACATTAGAGGATAACAAC
396
Signal Peptide
I F P K Q Y P I I N F T T A G A T V Q S Y T
ATATTCCCCAAACAATACCCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACACA
A-chain
Ban I
N F I R A V R G R L T T G A D V R H E I P V
AACTTTATCAGAGCTGTTCGCGGTCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTG
519
L P N R V G L P I N Q R F I L V E L S N H A
TTGCCAAACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCATGCA
594
E L S V T L A L D V T N A Y V V G Y R A G N
GAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATATGTGGTCGGCTACCGTGCTGGAAAT
660
S A Y F F H P D N Q E D A E A I T H L F T D
AGCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACTCATCTTTTCACTGAT
726
V Q N R Y T F A F G G N Y D R L E Q L A G N
GTTCAAAATCGATATACATTCGCCTTTGGTGGTAATTATGATAGACTTGAACAACTTGCTGGTAAT
792
L R E N I E L G N G P L E E A I S A L Y Y Y
CTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAGGCTATCTCAGCGCTTTATTATTAC
858
S T G G T Q L P T L A R S F I I C I Q M I S
AGTACTGGTGGCACTCAGCTTCCAACTCTGGCTCGTTCCTTTATAATTTGCATCCAAATGATTTCA
924
Fsp I
E A A R F Q Y I E G E M R T R I R Y N R R S
GAAGCAGCAAGATTCCAATATATTGAGGGAGAAATGCGCACGAGAATTAGGTACAACCGGAGATCT
990
A P D P S V I T L E N S W G R L S T A I Q E
GCACCAGATCCTAGCGTAATTACACTTGAGAATAGTTGGGGGAGACTTTCAACTGCAATTCAAGAG
1056
S N Q G A F A S P I Q L Q R R N G S K F S V
TCTAACCAAGGAGCCTTTGCTAGTCCAATTCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTG
1122
Fsp I
Y D V S I L I P I I A L M V Y R C A P P P S
TACGATGTGAGTATATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCG

FIG. 11-1

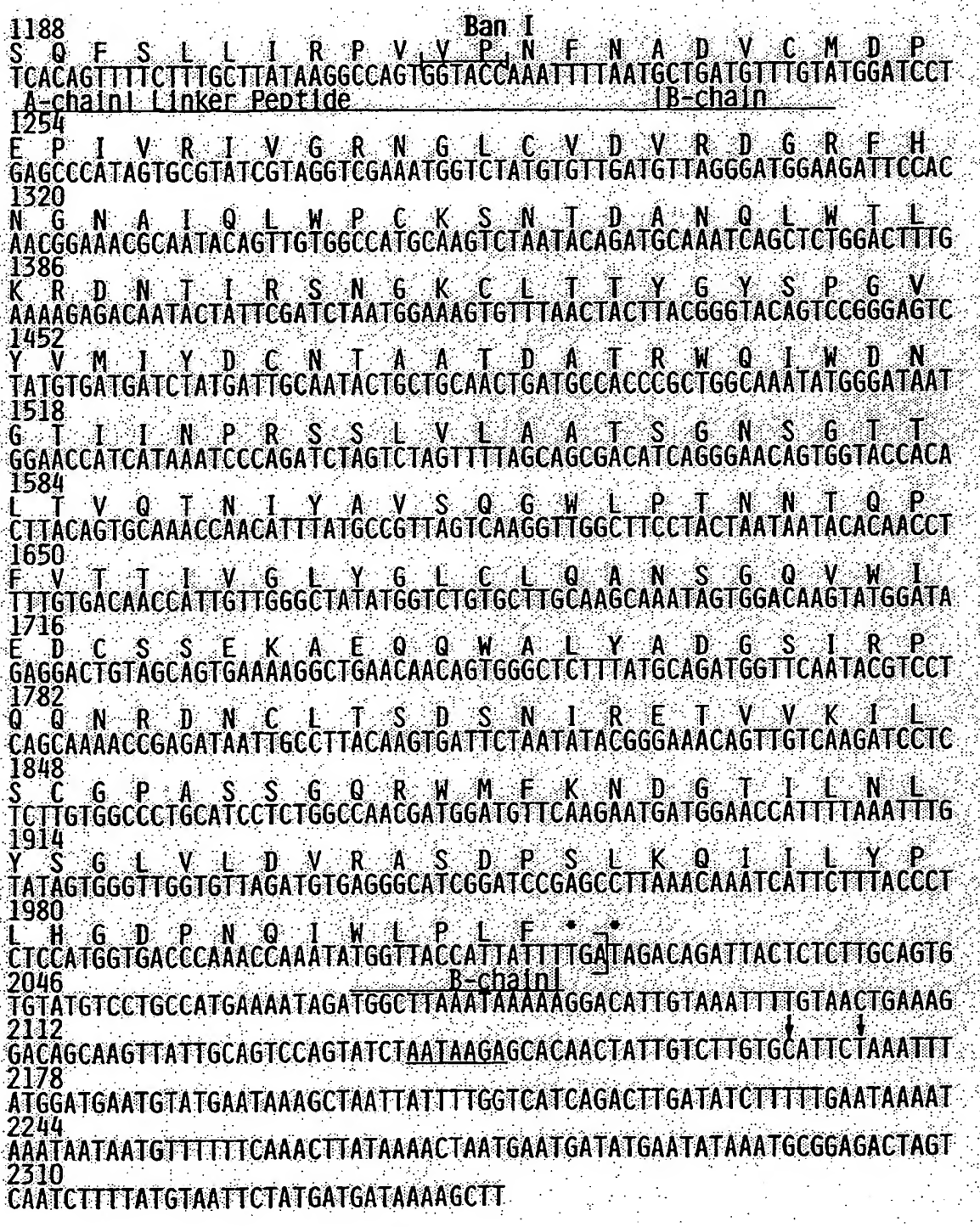


FIG. 11-2

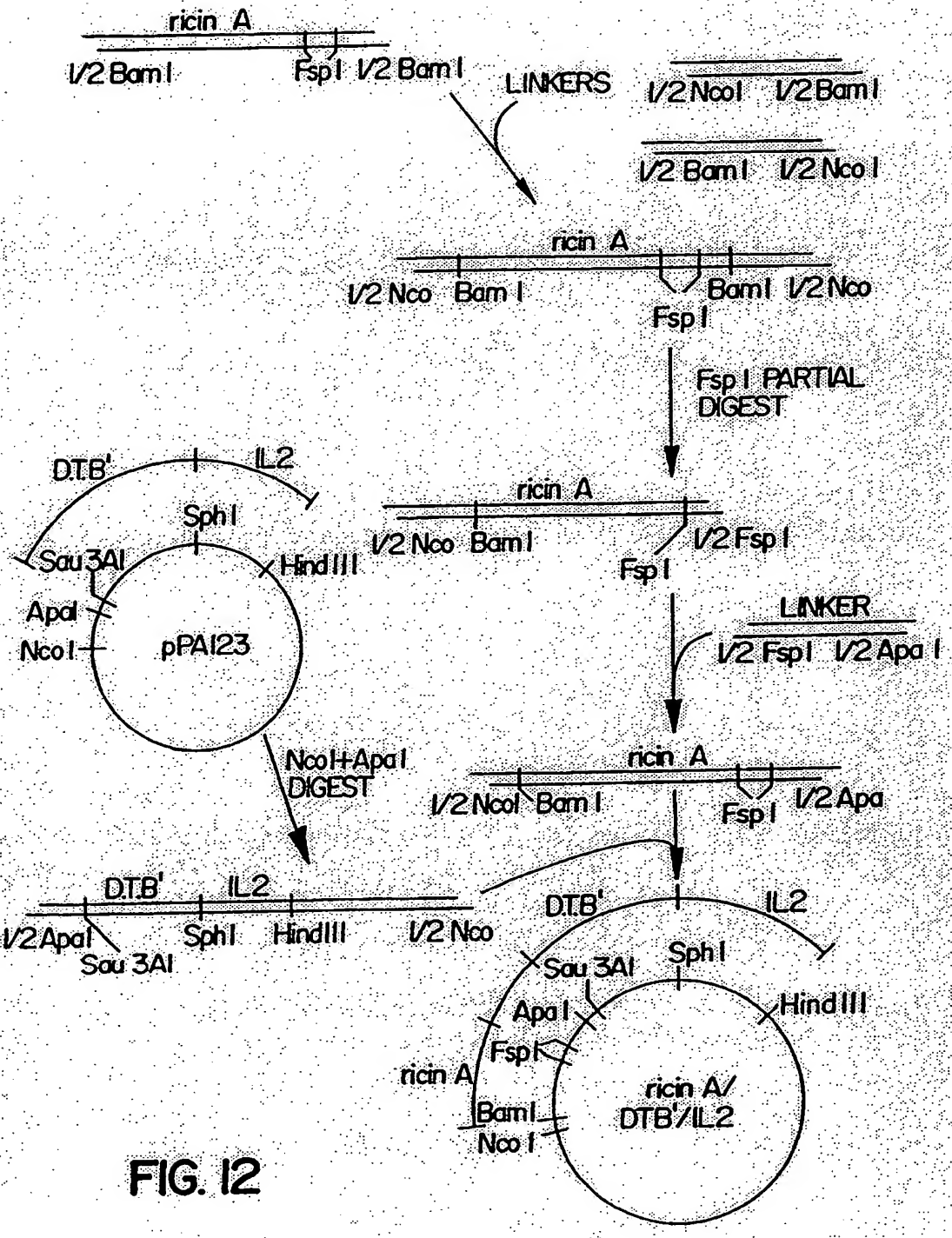

ricin A
1/2 Bam I
Fsp I
1/2 Bam I
LINKERS
1/2 Nco I
1/2 Bam I
1/2 Bam I
1/2 Nco I
ricin A
1/2 Nco
Bam I
Bam I
1/2 Nco
Fsp I
Fsp I PARTIAL DIGEST
DTB'
IL2
Sph I
Sau 3A I
Hind III
Apa I
Nco I
pPA123
ricin A
1/2 Nco
Bam I
Fsp I
1/2 Fsp I
LINKER
1/2 Fsp I
1/2 Apa I
Nco I + Apa I DIGEST
ricin A
1/2 Nco
Bam I
Fsp I
1/2 Apa
DTB'
IL2
1/2 Apa I
Sau 3A I
Sph I
Hind III
1/2 Nco
DTB'
IL2
Sau 3A I
Sph I
Apa I
Hind III
Fsp I
ricin A
ricin A/ DTB'/IL2
Bam I
Nco I

FIG. 12

```
                    15                  30                  45                  60                  75
CTT CAT CGT CGT CCA ACT GAC CTT GAG TGT TAG TTT CGC GGT AAG TTT GGG TAT AAG TGC CAC CAC CAG TGC CGG
                    90                 105                 120                 135                 150
CAG TGT AGT CAG TAG TTT GTT GCT GGA AGT CGT TAC CGC CAA ACT GCG TGT TAC CGC CTA GAT TAG ACT GTG GCT
                   165                 180                 195                 210                 225
GCT GGC GTT GAG GGA AAC CTG CCT GTA CGT GAG GCC CTA AAA AGC CAG AGA CCT CAC TCC CGG GGA GCC AGC ATG
                                                                                                Met
                   240                 255                 270                 285                 300
TCC ACT GCG GTC CTG GAA AAC CCA GGC TTG GGC AGG AAA CTC TCT GAC TTT GGA CAG GAA ACA AGC TAT ATT GAA
Ser Thr Ala Val Leu Glu Asn Pro Gly Leu Gly Arg Lys Leu Ser Asp Phe Gly Gln Glu Thr Ser Tyr Ile Glu
                   315                 330                 345                 360                 375
GAC AAC TGC AAT CAA AAT GGT GCC ATA TCA CTG ATC TTC TCA CTC AAA GAA GAA GTT GGT GCA TTG GCC AAA GTA
Asp Asn Cys Asn Gln Asn Gly Ala Ile Ser Leu Ile Phe Ser Leu Lys Glu Glu Val Gly Ala Leu Ala Lys Val
                   390                 405                 420                 435                 450
TTG CGC TTA TTT GAG GAG AAT GAT GTA AAC CTG ACC CAC ATT GAA TCT AGA CCT TCT CGT TTA AAG AAA GAT GAG
Leu Arg Leu Phe Glu Glu Asn Asp Val Asn Leu Thr His Ile Glu Ser Arg Pro Ser Arg Leu Lys Lys Asp Glu
                   465                 480                 495                 510                 525
TAT GAA TTT TTC ACC CAT TTG GAT AAA CGT AGC CTG CCT GCT CTG ACA AAC ATC ATC AAG ATC TTG AGG CAT GAC
Tyr Glu Phe Phe Thr His Leu Asp Lys Arg Ser Leu Pro Ala Leu Thr Asn Ile Ile Lys Il  Leu Arg His Asp
                   540                 555                 570                 585                 600
ATT GGT GCC ACT GTC CAT GAG CTT TCA CGA GAT AAG AAG AAA GAC ACA GTG CCC TGG TTC CCA AGA ACC ATT CAA
Ile Gly Ala Thr Val His Glu Leu Ser Arg Asp Lys Lys Lys Asp Thr Val Pro Trp Phe Pro Arg Thr Ile Gln
                   615                 630                 645                 660                 675
GAG CTG GAC AGA TTT GCC AAT CAG ATT CTC AGC TAT GGA GCG GAA CTG GAT GCT GAC CAC CCT GGT TTT AAA GAT
Glu Leu Asp Arg Phe Ala Asn Gln Ile Leu Ser Tyr Gly Ala Glu Leu Asp Ala Asp His Pro Gly Phe Lys Asp
                   690                 705                 720                 735                 750
CCT GTG TAC CGT GCA AGA CGG AAG CAG TTT GCT GAC ATT GCC TAC AAC TAC CGC CAT GGG CAG CCC ATC CCT CGA
Pro Val Tyr Arg Ala Arg Arg Lys Gln Phe Ala Asp Ile Ala Tyr Asn Tyr Arg His Gly Gln Pro Ile Pro Arg
                   765                 780                 795                 810                 825
GTG GAA TAC ATG GAG GAA GAA AAG AAA ACA TGG GGC ACA GTG TTC AAG ACT CTG AAG TCC TTG TAT AAA ACC CAT
Val Glu Tyr Met Glu Glu Glu Lys Lys Thr Trp Gly Thr Val Phe Lys Thr Leu Lys Ser Leu Tyr Lys Thr His
                   840                 855                 870                 885                 900
GCT TGC TAT GAG TAC AAT CAC ATT TTT CCA CTT CTT GAA AAG TAC TGT GGC TTC CAT GAA GAT AAC ATT CCC CAG
Ala Cys Tyr Glu Tyr Asn His Ile Phe Pro Leu Leu Glu Lys Tyr Cys Gly Phe His Glu Asp Asn Ile Pro Gln
```

FIG. 13-1

```
                915                 930                 945                 960                 975
CTG GAA GAC GTT TCT CAA TTC CTG CAG ACT TGC ACT GGT TTC CGC CTC CGA CCT GTG GCT GGC CTG CTT TCC TCT
Leu Glu Asp Val Ser Gln Phe Leu Gln Thr Cys Thr Gly Phe Arg Leu Arg Pro Val Ala Gly Leu Leu Ser Ser
                990                1005                1020                1035                1050
CGG GAT TTC TTG GGT GGC CTG GCC TTC CGA GTC TTC CAC TGC ACA CAG TAC ATC AGA CAT GGA TCC AAG CCC ATG
Arg Asp Phe Leu Gly Gly Leu Ala Phe Arg Val Phe His Cys Thr Gln Tyr Ile Arg His Gly Ser Lys Pro Met
               1065                1080                1095                1110                1125
TAT ACC CCC GAA CCT GAC ATC TGC CAT GAG CTG TTG GGA CAT GTG CCC TTG TTT TCA GAT CGC AGC TTT GCC CAG
Tyr Thr Pro Glu Pro Asp Ile Cys His Glu Leu Leu Gly His Val Pro Leu Phe Ser Asp Arg Ser Phe Ala Gln
               1140                1155                1170                1185                1200
TTT TCC CAG GAA ATT GGC CTT GCC TCT CTG GGT GCA CCT GAT GAA TAC ATT GAA AAG CTC GCC ACA ATT TAC TGG
Phe Ser Gln Glu Ile Gly Leu Ala Ser Leu Gly Ala Pro Asp Glu Tyr Ile Glu Lys Leu Ala Thr Ile Tyr Trp
               1215                1230                1245                1260                1275
TTT ACT GTG GAG TTT GGG CTC TGC AAA CAA GGA GAC TCC ATA AAG GCA TAT GGT GCT GGG CTC CTG TCA TCC TTT
Phe Thr Val Glu Phe Gly Leu Cys Lys Gln Gly Asp Ser Ile Lys Ala Tyr Gly Ala Gly Leu Leu Ser Ser Phe
               1290                1305                1320                1335                1350
GGT GAA TTA CAG TAC TGC TTA TCA GAG AAG CCA AAG CTT CTC CCC CTG GAG CTG GAG AAG ACA GCC ATC CAA AAT
Gly Glu Leu Gln Tyr Cys Leu Ser Glu Lys Pro Lys Leu Leu Pro Leu Glu Leu Glu Lys Thr Ala Ile Gln Asn
               1365                1380                1395                1410                1425
TAC ACT GTC ACG GAG TTC CAG CCC CTG TAT TAC GTG GCA GAG AGT TTT AAT GAT GCC AAG GAG AAA GTA AGG AAC
Tyr Thr Val Thr Glu Phe Gln Pro Leu Tyr Tyr Val Ala Glu Ser Phe Asn Asp Ala Lys Glu Lys Val Arg Asn
               1440                1455                1470                1485                1500
TTT GCT GCC ACA ATA CCT CGG CCC TTC TCA GTT CGC TAC GAC CCA TAC ACC CAA AGG ATT GAG GTC TTG GAC AAT
Phe Ala Ala Thr Ile Pro Arg Pro Phe Ser Val Arg Tyr Asp Pro Tyr Thr Gln Arg Ile Glu Val Leu Asp Asn
               1515                1530                1545                1560                1575
ACC CAG CAG CTT AAG ATT TTG GCT GAT TCC ATT AAC AGT GAA ATT GGA ATC CTT TGC AGT GCC CTC CAG AAA ATA
Thr Gln Gln Leu Lys Ile Leu Ala Asp Ser Ile Asn Ser Glu Ile Gly Ile Leu Cys Ser Ala Leu Gln Lys Ile
               1590                1605                1620                1635                1650
AAG TAA AGC CAT GGA CAG AAT GTG GTC TGT CAG CTG TGA ATC TGT TGA TGG AGA TCC AAC TAT TTC TTT CAT CAG
Lys ***
               1665                1680                1695                1710                1725
AAA AAG TCC GAA AAG CAA ACC TTA ATT TGA AAT AAC AGC CTT AAA TCC TTT ACA AGA TGG AGA AAC AAC AAA TAA
               1740                1755                1770                1785                1800
GTC AAA ATA ATC TGA AAT GAC AGG ATA TGA GTA CAT ACT CAA GAG CAT AAT GGT AAA TCT TTT GGG GTC ATC TTT
               1815                1830                1845                1860                1875
GAT TTA GAG ATG ATA ATC CCA TAC TCT CAA TTG AGT TAA ATC AGT AAT CTG TCG CAT TTC ATC AAG ATT AAT TAA
```

FIG. 13-2

```
                1890                1905                1920                1935                1950
AAT TTG GGA CCT GCT TCA TTC AAG CTT CAT ATA TGC TTT GCA GAG AAC TCA TAA AGG AGC ATA TAA GGC TAA ATG
                1965                1980                1995                2010                2025
TAA AAC ACA AGA CTG TCA TTA GAA TTG AAT TAT TGG GCT TAA TAT AAA TCG TAA CCT ATG AAG TTT ATT TTC TAT
                2040                2055                2070                2085                2100
TTT AGT TAA CTA TGA TTC CAA TTA CTA CTT TGT TAT TGT ACC TAA GTA AAT TTT CTT TAG GTC AGA AGC CCA TTA
                2115                2130                2145                2160                2175
AAA TAG TTA CAA GCA TTG AAC TTC TTT AGT ATT ATA TTA ATA TAA AAA CAT TTT TGT ATG TTT TAT TGT AAT CAT
                2190                2205                2220                2235                2250
AAA TAC TGC TGT ATA AGG TAA TAA AAC TCT GCA CCT AAT CCC CAT AAC TTC CAG TAT CAT TTT CCA ATT AAT TAT
                2265                2280                2295                2310                2325
CAA GTC TGT TTT GGG AAA CAC TTT GAG GAC ATT TAT GAT GCA GCA GAT GTT GAC TAA AGG CTT GGT TGG TAG ATA
                2340                2355                2370                2385                2400
TTC AGG AAA TGT TCA CTG AAT AAA TAA GTA AAT ACA TTA TTG AAA AGC AAA TCT GTA TAA ATG TGA AAT TTT TAT
                2415                2430                2445
TTG TAT TAG TAA TAA AAC ATT AGT AGT TTA AAA AAA AAA AAA AAA AAA
```

FIG. 13-3

EP 0 439 954 A2

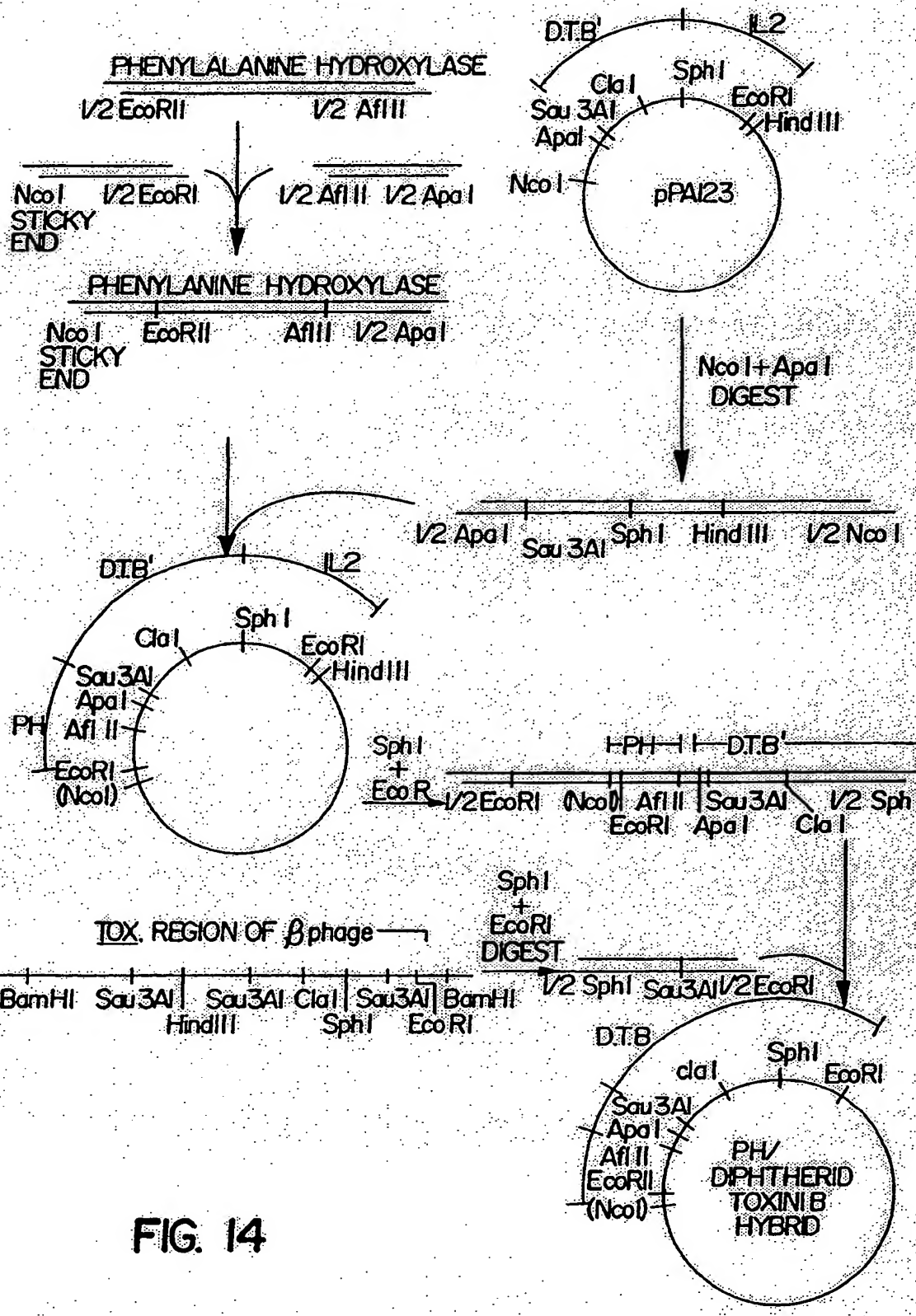

PHENYLALANINE HYDROXYLASE
1/2 EcoRII
1/2 AflII
Nco I
1/2 EcoRI
STICKY END
1/2 AflII
1/2 Apa I
PHENYLANINE HYDROXYLASE
Nco I
EcoRII
AflII
1/2 Apa I
STICKY END
D.T.B'
IL2
Cla I
Sph I
EcoRI
Hind III
Sau 3AI
Apa I
Nco I
pPA123
Nco I + Apa I DIGEST
1/2 Apa I
Sau 3AI
Sph I
Hind III
1/2 Nco I
D.T.B'
IL2
Sph I
Cla I
EcoRI
Hind III
Sau3AI
Apa I
PH
Afl II
EcoRI
(Nco I)
Sph I + EcoR
PH
D.T.B'
1/2 EcoRI
(Nco I)
Afl II
Sau3AI
1/2 Sph I
EcoRI
Apa I
Cla I
TOX. REGION OF β phage
BamHI
Sau3AI
Sau3AI
Cla I
Sau3AI
BamHI
HindIII
Sph I
EcoRI
Sph I + EcoRI DIGEST
1/2 Sph I
Sau3AI
1/2 EcoRI
D.T.B
Sph I
cla I
EcoRI
Sau3AI
Apa I
Afl II
EcoRII
(Nco I)
PH/ DIPHTHERID TOXIN I B HYBRD

FIG. 14